CYSTIC FIBROSIS MICROBIOLOGY: MOLECULAR FINGERPRINTING OF MICROBIAL PATHOGENS

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This thesis is dedicated to my daughter Lynsey and in memory of my son Liam.

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ABBREVIATIONS

AAV	Adeno-associated virus
AP-PCR	Arbitrarily primed polymerase chain reaction
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CDC	Communicable Disease Center
cDNA	Cyclic deoxyribonucleic acid
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
cfu/ml	Colony forming units per millilitre
CMV	Cytomegalovirus
CP	Capsular polysaccharide
DOPE	Dioleoylphosphatidylethanolamine
EET	Esterase electrophoretic typing
ELISA	Enzyme-linked immunosorbent assay
ERIC	Enterobacterial repetitive intergenic consensus sequence
ESR	Erythrocyte sedimention rate
ETA	Exotoxin A
FIGE	Field-inversion gel electrophoresis
IS	Insertion sequence
kb	Kilobase
LDC	Lysine decarboxylase
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
MLEE	Multilocus enzyme electrophoresis
MOMP	Major outer membrane protein
mRNA	Messenger ribosomal nucleic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
N/S	Nose swab
ODC	Ornithine decarboxylase
OMP	Outer membrane protein
ONPG	*o*-nitrophenyl-β-D-galactopyranoside

PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RSV	Respiratory syncytial virus
TD	Treatment day
T/S	Throat swab
WBC	White blood cell

ABSTRACT

Cystic fibrosis (CF) is the most common, and ultimately fatal, inherited disease of Caucasian populations with an incidence of 1 in 2,500 live births and affecting over 50,000 children and adults world-wide. Progressive lung disease, resulting from impaired mucociliary clearance and persistent bacterial infection of the bronchial mucosa, is the main cause of morbidity and mortality and affects over 90% of individuals with CF.

CF lung infections are caused by a surprisingly narrow spectrum of pathogens and include *Staphylococcus aureus, non-capsulate Haemophilus influenzae, Pseudomonas aeruginosa and Burkholderia cepacia. Stenotrophomonas maltophila* is recovered from respiratory secretions with increasing frequency, however, its pathogenic role remains unclear. The primary aims of this thesis include the development and use of genomic fingerprinting systems to assist epidemiological investigations of CF pathogens, including *S.maltophilia.*

Genomic fingerprinting is based on digestion of total bacterial chromosomal DNA with rare cutting enzymes, chosen on the basis of the bacterium's GC content. Separation of the DNA fragments is then achieved by pulsed-field gel electrophoresis (PFGE) in an appropriate apparatus such as the Bio-Rad contour clamped homogenous electric field (CHEF) system. Although a variety of other genomic typing systems are available, the thesis focused on PFGE, potentially the most discriminating system at present. Another major theme of the thesis concerned the

epidemiology of *B.cepacia.* This highly adaptable plant and human pathogen causes great anxiety in the CF community on account of its inherent resistance, transmissibility and association with cepacia syndrome, a rapidly fatal pneumonia affecting approximately 30% of colonised patients. PFGE is technically demanding, time consuming and relatively expensive, thus attempts were made to assess the reliability and potential of other systems, in particular, PCR-ribotyping as a simple and rapid screening system for clonal analyses.

The project provided a limited opportunity for fingerprinting and other microbiological studies of the commensal and pathogenic respiratory flora in CF patients participating in the first human trials of CF gene therapy. Specimens were examined before, during and after local nasal administration of a DNA/liposome complex. Although only a Phase I study was achieved during the duration of the thesis, microbiological analyses provided interesting results, in particular an unexpected lack of clonal relationship between *S. aureus* colonising the upper and lower airways.

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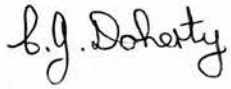
DECLARATION

All of the experiments and procedures presented in this thesis were carried out by the author unless otherwise stated.



CHAPTER 1

CYSTIC FIBROSIS

Cystic fibrosis (CF), otherwise known as mucoviscidosis or fibrocystic disease of the pancreas, was first recognised as a fatal disease of infants in the late 1930's (Andersen, 1938). Today, it is recognised as the most common, fatal autosomal recessive disease among Caucasian populations, although it is not found exclusively in Caucasians. With an incidence of approximately 1 in 2,500 live births, CF affects around 6,000 individuals in the UK and about 600 in Scotland. The incidence, however, varies considerably on a geographical basis from 1 in 1,700 in N. Ireland, 1 in 7,700 in Sweden to 1 in 17,000 in African-Americans (Bye *et al*, 1994) and reaches the highest level (1 in 313) in the closely-knit populations such as the Hutterites of North America (Mickle & Cutting, 1998).

One in 25 of normal individuals are heterozygous CF carriers. Carrier status is asymptomatic but when both parents are carriers there is a 1 in 4 chance in every pregnancy of a CF infant.

1.1 CLINICAL FEATURES

CF is a multisystem disorder of the exocrine glands including the lung, gastrointestinal tract, pancreas, liver, vas deferans and the sweat glands. The major pathophysiological aspects of the disease were described by Andersen (1938) as

pancreatic insufficiency with malnutrition, hepatic disease and infection of the airways. CF patients produce extremely viscous secretions which leads to autodigestion of the pancreas through blockage of the pancreatic ducts and inability to secrete digestive enzymes. Pancreatic insufficiency, occuring in 85 - 90% of CF patients, leads to malabsorption of fat and proteins but this is largely corrected by the use of oral pancreatic enzyme supplements (Kopelman, 1991). An additional complication of CF, generally restricted to patients with pancreatic insufficiency, is diabetes mellitis which occurs in 10-12% of patients with a tendancy to increase with increasing age (Shwachman & Holsclaw, 1969).

Hepatic disease was described in the initial pathologic descriptions of CF (Andersen, 1938). The most common finding is focal biliary cirrhosis (Webster & Williams, 1953) found in less than 25% of CF patients while the more serious multilobulated biliary cirrhosis occurs in 2-5% (di Sant' Agnese & Blanc, 1956) and may result in progressive clinical liver failure. Symptomatic liver disease has been successfully treated with implanted shunts or transplantation.

Meconium ileus is one of the earliest clinical manifestation of CF and occurs in 10-20% of patients. Historically, it was a common cause of infant mortality but is now a rare cause of death due to early diagnosis of CF, improved paediatric surgery and neonatal intensive care (Dodge *et al*, 1988)

Genitourinary abnormalities are found in both sexes although more common in males. Male sterility is due to obstruction of the vas deferens and occurs in 95% of male CF patients. Decreased fertility in females occurs due to increased amount of mucus in the cervical canal resulting in blockages by mucus plugs (Bye *et al*, 1994).

Increased viscous secretions in CF patients also cause airway obstruction resulting in impaired mucociliary clearance and recurrent pulmonary infections. Lung infection and consequent bronchial and alveolar damage are the main causes of morbidity and mortality in CF patients and are responsible for about 90% of deaths (Hoiby, 1982; Fick, 1989). Progressive decline in pulmonary function is due to a viscous cycle of airway obstruction, infection and inflammation. Airway inflammation is demonstrated by raised levels of neutrophil and bacteria DNA, interleukin-8, free neutrophil elastase and neutrophils in bronchoalveolar lavage fluid (Khan *et al*, 1995). These inflammatory markers may be present before the onset of clinically apparent pulmonary disease.

1.2 CF MICROBIOLOGY

Surprisingly, only a small spectrum of microbial pathogens, mostly bacteria, are responsible for causing pulmonary infection in individuals with CF. Typically, initial infection is caused by *Staphylococcus aureus* and *Haemophilus influenzae* with *Pseudomonas aeruginosa* appearing at a later stage. *Burkholderia cepacia* is a more recent pathogen in CF; colonisation with this inherently resistant environmental bacterium is a cause of great concern among the CF community as certain strains are

readily transmissible from patient-to-patient and approximately 30% of colonised patients succumb to rapidly fatal pneumonia. In recent years, another potential pathogen *Stenotrophomonas maltophilia* has emerged with increasing incidence although its clinical role is unclear. Each of these major CF pathogens will be discussed individually in later chapters of this thesis.

Other microorganisms are occasionally isolated from the respiratory secretions of CF patients but they do not appear to persist for extended periods of time. These include *Streptococcus pneumoniae*, enteric bacilli and Gram-negative glucose-nonfermenting bacilli other than *P. aeruginosa* and *B. cepacia.* Another two uncommon respiratory organisms, *Alcaligenes xylosoxidans* (Vu Thien *et al*, 1996; Karpati, *et al*, 1994) and *Burkholderia gladioli* (Wilsher *et al*, 1997) are increasingly isolated from sputum specimens from CF patients.

Mycobacterium tuberculosis is rarely a cause of infection in CF patients and when it does occur it can be treated successfully with anti-mycobacterium antibiotics (Gilligan, 1991). Rapid growing *Mycobacterium* species such as *M. avium* and *M. fortuitum* have been recovered from respiratory secretions of CF patients and although they play a minor role in lung disease they may contribute to lung deterioration (Boxerbaum, 1980; Gilligan, 1991).

Aspergillus fumigatus is the major fungal agent associated with pulmonary infection in CF patients, causing allergic bronchopulmonary aspergillosis (ABA). The

prevalence of ABA ranges from 0.5 - 11% and is thought to be more prevalent in older children and young adults (Brueton *et al*, 1980; Paul *et al*, 1996). In CF patients, clinical symptoms must be considered carefully in the diagnosis of ABA; the prevalence of clinical disease is low despite the frequent presence of immunologic responses to *A. fumigatus* colonisation (Becker *et al*, 1996). The yeast, *Candida albicans* is frequently recovered from the respiratory tract of CF patients, especially those receiving antibiotics or steroids, and usually disappears after treatment is completed.

The role of viruses in CF lung disease is still unclear. Respiratory syncytial virus (RSV) may act as a predisposing factor to bacterial colonisation with *P. aeruginosa* (Petersen *et al*, 1981). RSV may also be responsible for deterioration in lung function in young CF children (Abman *et al*, 1988). The role of other viruses, including influenza and parainfluenza, in lower respiratory tract infection is similar in CF as to that in non-CF individuals. Infection with *Chlamydia pneumoniae* has been associated with acute pulmonary exacerbations in some CF patients (Emre *et al*, 1996).

The life expectancy of children with CF has increased dramatically in recent years. In the early 1930's, a CF child rarely lived beyond infancy and even in 1969 the mean survival age was only 14 years (FitzSimmons, 1993). In contrast, CF patients born in the 1990's, have a projected mean survival age of 40 years (Collins, 1992). A number of factors are thought to be associated with increased life expectancy. Early

diagnosis of the disease has been associated with improved nutritional status, decreased morbidity and less deterioration in lung function. However, there is no evidence that early detection of CF through neonatal screening has led to an improvement in survival (Rosenstein & Zeitlin, 1998). However, the establishment of large CF centres throughout the UK has provided multidisciplinary management of pulmonary and nutritional status of the patient. Despite the promise of gene therapy, at present antimicrobial therapy is the major therapeutic intervention to reduce pulmonary infection and hence increase life expectancy. Other factors include lung or heart/lung transplantation with long term survival rates of 5 years and over in 60 - 65% of patients. Recently, bilateral lobar transplantation, from related donors, has been attempted successfully (Moss, 1995). As with all transplants, there are problems of rejection and organ availability. In CF patients, however, colonisation with *Mycobacterium* species or *B. cepacia* may also result in exclusion from transplantation lists at some units.

Recent therapeutic strategies in CF include the use of recombinant deoxyribonuclease to reduce the viscoelasticity of CF sputum. This relatively new treatment appears to benefit some patients in conjunction with conventional treatment by reducing the frequency of pulmonary exacerbations and delaying the decline in pulmonary function (Fuchs *et al*, 1994). Expanding knowledge of naturally-occuring antimicrobial peptides (defensins) in pulmonary secretions and their relevance to CF lung infection may provide another potential treatment against bacterial infections. These antimicrobial peptides are produced in human or animal

airways; of particular relevance some defensins are 'salt-sensitive'. It has been proposed that inactivation of defensins by high concentrations of salt explains why CF patients become chronically colonised with bacterial pathogens such as *P. aeruginosa* (Goldman *et al*, 1997).

1.3 DIAGNOSIS OF CF

One of the characteristic features of CF, affecting over 95% of patients, is elevated levels of sweat electrolytes. This sweat abnormality led to the development of the sweat test, which relies on detection of higher than normal concentrations of sodium and chlorine in sweat (McPherson & Dormer, 1987). Although reasonably reliable screening methods for sweat testing are available, results should be confirmed by quantitative pilocarpine iontophoresis Gibson & Cooke, 1959).

Neonatal screening procedures may detect CF but as yet these are not carried out routinely in all newborn babies. Immunoreactive trypsin is first measured in dried blood spots; if the trypsin level is high, the sample is tested for the most common CFTR mutation, ΔF508, or a panel of common mutations (Wilcken *et al*, 1995). CF can also be diagnosed in utero by detection of two CF mutations in the foetus by chorionic villus sampling or amniocentesis; in general, such testing is only done when there is a history of CF in the family. The discovery of the CF gene in 1989 has made prenatal diagnosis of CF more accurate and accessible. It has also led to improved accuracy in the diagnosis of CF, especially in patients with unusual CF phenotypes (Strong *et al*, 1991).

1.4 DISCOVERY OF CF GENE

In 1985, following location of the CF gene on the long arm of chromosome 7 at position 7q31-32 (Tsui *et al*, 1985), the race to sequence the gene and identify the encoded protein accelerated. This achievement one of the most outstanding in modern biomedical science, occured in 1989 when the CF gene was cloned and sequenced using genetic linkage analysis and techniques of chromosome walking and jumping (Rommens *et al*, 1989; Riordan *et al*, 1989; Kerem *et al*, 1989). The 250kb gene encodes a transmembrane protein, the cystic fibrosis transmembrane conductance regulator (CFTR) comprising 1,480 amino acids. CF results from mutations in the development or functions of CFTR and to date over 700 mutations have been described. ΔF508 is the most common and is found in approximately 70% of CF patients; this mutation results in a three base pair deletion resulting in loss of phenylalanine at position 508 in the amino acid sequence.

1.5 ROLE OF CFTR

CFTR functions as a cAMP-regulated chloride (Cl) channel in the apical membrane of epithelial cells (Welsh, 1990). In non-CF lungs, increases in intracellular cAMP result in increased Cl^- transport followed by the movement of water by osmosis and maintenance of adequately hydrated mucociliary clearance. In CF, CFTR is mislocated or dysfunctional resulting in an alteration in the regulation of ion transport across the apical membranes of the epithelial cells in the airways, gut, pancreas, biliary and sperm ducts. In 1982, Quinton, a pharmacologist who himself

has CF, was the first to note that the CF epithelium is relatively impermeable to Cl^-, suggesting a primary defect in anion transport (Quinton, 1983).

In the sweat glands of normal individuals , the Cl^- transport by epithelial cells of the secretory coil, is accomplished by two channels, CFTR and a calcium-activated Cl channel: acting together to produce isotonic fluid. As this fluid flows the epithelial cells lining the duct lumen absorb sodium (Na) and Cl ions producing hypotonic sweat. Both mechanisms are dependant on CFTR. In CF, these Cl channels fail to open resulting in high residual Cl^- and Na^+ concentrations in the sweat. The opposite occurs in the respiratory tract; Cl^- transport from within the cells towards the lumen of the bronchus is impaired. The transport of Na^+ is also abnormal in respiratory epithelia as there is an increase absorption of Na from the lumen into the cells and diffusion of water from interstitial fluid into the lumen is reduced (Knowles *et al*, 1981). These abnormalities in ion transport and hydration are thought to result in an increase in viscosity of respiratory mucus which leads to impaired mucociliary clearance predisposing to recurrent repiratory infections. Despite major advances in the genetics and molecular basis of CF, the pathogenesis of lung disease remains unclear and highly controversial.

Studies of cultured human airway epithelial cells from CF and non-CF patients have shown that airway surface liquid (ASL) covering CF epithelia have Na^+ and Cl^- concentrations that were approximately double the value of non-CF epithelia (Zabner *et al*, 1998). In contrast, two other groups found no difference in the ASL NaCl

concentrations between CF and non-CF patients (Knowles *et al*, 1997; Hull *et al*,1998)

A recent study by Matsui *et al* (1998) using planar and cylindrical culture models also showed that there was little difference in the NaCl concentration of ASL of CF and non-CF patients. However, the rate of ASL adsorption in CF respiratory epithelia was abnormally high compared to normal respiratory epithelia. This accelerated adsorption of ASL in CF resulted in depletion of the periciliary liquid layer and concentration of the mucus on airway surfaces. The authors speculated that failure of the thickened mucus to be removed from the airway surfaces allows bacteria to colonise resulting in pulmonary infection. If this hypothesis is correct, a logical therapeutic strategy treatment would be to add isotonic fluid to respiratory surfaces to restore the fluid volume in the periciliary layer.

1.6 PROTEIN-REPAIR THERAPY

The different mutations of CFTR, responsible for causing CF, have been characterised into four classes I - IV, based on biochemical disfunction (Welsh & Smith, 1993) and a fifth group identified by Gan *et al* in 1995 was designated class V by Rosenstein & Zeitlin (1998). An improved understanding of these different mutations found in CFTR has led to new therapeutic approaches, termed protein-repair therapy.

Class I mutations cause defective CFTR protein production with little or no full-length protein being produced and result in a loss of CFTR chloride ion channel function in affected epithelia. Such mutations may involve frameshifts due to insertions and deletions, splice site abnormalities and nonsense mutations (Tsui, 1992) and in some cases mRNAs are unstable (Hamosh *et al*, 1991). However, certain aminoglycosides, such as gentamicin, can suppress these mutations and restore full-length CFTR mRNA protein to the cell.

Class II mutations result in defective protein processing. ΔF508 is the most common mutation in this class. The defect causes structural assembly in the endoplasmic reticulum to be inefficiently glycosylated or transported to the correct cellular location resulting in degradation of the CFTR protein (Cheng *et al*, 1990). Other mutations, in this group, may be processed through the Golgi complex but fail to be transported to the apical membrane.

Class III mutations are completely defective with respect to regulation and activation of chloride channel activity. Although some mutant proteins do reach the plasma membrane they have mutations in the nucleotide-binding domains. These mutations can alter channel function as intracellular ATP regulates the opening of CFTR chloride channels through direct interactions with the nucleotide-binding domains. Some nucleotide-binding domain mutants have very little function, in others ATP is less potent at stimulating activity (Anderson & Welsh, 1992; Drumm *et al*, 1991). CFTR is also regulated by phosphorylation of the regulatory domain and although

mutants have been reported in the regulatory domain they are less common than in other parts of the protein (Welsh & Smith, 1993).

Class IV mutations demonstrate normal regulation but altered conduction. Channel regulation appears intact but mutations in the membrane-spanning domain reduce the rate of Cl^- flow through the open channel (Welsh & Smith, 1993).

Mutations in classes III or IV are unresponsive to direct stimulation and protein-repair would be of little use, however, gene therapy may be a potential treatment.

Class V mutations show reduced synthesis of CFTR and are found in patients who tend to have milder lung disease, borderline or normal sweat electrolyte values and residual chloride secretion in the intestine.

Patients with defective CFTR in classes I - III generally exhibit pancreatic insufficiency while those with class IV and V defects are pancreatic sufficient. At present, there does not appear to be a correlation between the classification of CFTR mutations and the pulmonary phenotype with the exception of ΔF508 and increased *Pseudomonas aeruginosa* colonisation.

1.7 GENE THERAPY

Since the development of recombinant DNA technology in the 1970's, the prospect that genetic diseases could be treated by introducing normal genes into affected

individuals has been a possibility. Gene therapy for CF will require direct *in vivo* delivery of vectors, containing the normal CFTR gene, into somatic cells of the host which should then express the gene as messenger RNA (mRNA) and manufacture normal CFTR. It is hoped that this will lead to normal ion channel functioning and thus correction of the fundamental defect causing the disease. As with any new therapy, thorough testing in cell culture and animal models is essential to monitor the safety and efficacy of the vectors prior to the application of gene therapy to the patient.

Several *in vitro* studies, using different cell lines, have shown interesting results. Correction of the CF Cl^- permeability defect has been demonstrated in CF pancreatic cells (Rosenfeld *et al*, 1992) and in CF epithelial cells using retrovirus and vaccinia virus vectors (Drumm *et al*, 1990; Rich *et al*,1990).

Extensive *in vivo* studies in animals have been undertaken using both the principal gene delivery systems of modified adenovirus and cationic liposomes. The first successful *in vivo* experiments involved transfer of the human CFTR gene, by means of a modified adenovirus, into the respiratory tract of cotton rats (Rosenfeld *et al*, 1992); this resulted in detection of mRNA and CFTR protein within a week and was sustained for up to four weeks. The development of a genetically modified mouse model of CF, which is genotypically and phenotypically similar to human CF, should also help answer some of the problems of gene therapy. Experiments using this murine model showed that when the gene expressing CFTR is completely removed

(Snouwaert *et al*,1992) or even when low levels of wild type CFTR mRNA are produced (Davidson *et al*, 1995), CF mice demonstrate gut and pulmonary disease abnormalities similar to CF patients. In the Edinburgh CF mutant mouse model the abnormal tracheal potential difference associated with CF was partially or wholly corrected by instillation (Hyde *et al*, 1993) or aerosolisation (McLachlan *et al*, 1996; Alton *et al*, 1993) of human cDNA complexed with cationic liposomes. Grubb and colleagues have also demonstrated a partial correction of the Cl^- transport defect in the nasal epithelium of the CF mice following *in vivo* adeno-mediated gene transfer to the nasal epithelium. However, no correction of Na^+ transport was observed. An *in vivo* study using adenovirus-mediated gene transfer to the airway epithelium of primates showed low efficiency (Rich *et al*, 1993). These animal experiments into CF gene therapy were swiftly followed by a number of Phase1 clinical trials in humans.

1.7.1 Human gene therapy for CF

Which cells to target ?

Although the mutation in CFTR affects a number of different cell types in the body the most important gene therapy target is considered to be the respiratory tract as this is the main organ system associated with CF disease and mortality. The highest levels of CFTR are expressed in the submucosal glands while much reduced levels are found in the surface epithelium. Furthermore, there are about 40 different cell types within the respiratory tract and it is still not clear which cells offer the best target. As the overall expression of CFTR in the lungs is low, it may be that clinical

benefit can be achieved by correcting a small percentage of the target cells in order to normalise the Cl conductance (Johnson *et al*, 1992). This is encouraging as *in vivo* studies have shown relatively low delivery using present vectors.

What vectors to use?

Currently, two main delivery systems are under investigation for transfer of the CF gene into target cells; these are modified adenovirus and cationic liposomes. Recombinant adenovirus vectors were originally favoured for gene delivery to the CF lung because of their natural airway tropism, capacity to transduce the human CFTR gene and because of encouraging results achieved with these viral vectors both *in vitro* and *in vivo*. The adenovirus is modified for gene therapy by deleting and inactivating the gene responsible for replication. Currently adenoviruses of serotype 2 and 5 are employed as vectors; in each case the modification involves the deletion of the early region one (E1) and a further deletion in the early region three (E3) in serotype 5 (Engelhardt *et al*, 1993). Deletions of the E1 region results in a non-replicating virus and the E3 region creates adequate space to insert the CFTR gene with a suitable promotor to drive transcription. The recombinant 4.5kb human CFTR cDNA, is then incorporated into the viral genome and delivered to the target cell where it enters by a process of receptor-mediated endocytosis (Fitzgerald *et al*, 1983). Adenoviruses have an innate ability to escape the endolysosomal pathway and exist primarily as an episome and seldom integrate into the host cell genome. Unfortunately, gene therapy trials involving the use of adenovirus as a vector have been associated with an inflammatory response (Crystal *et al*, 1994).

The second delivery system for gene therapy, employed mainly in the UK, involves the use of cationic liposomes. Liposomes are an attractive vector for gene delivery because they are chemically defined, readily manufactured to pharmaceutical standards and biodegradable. As used in the Brompton trial, cationic liposomes are composed of cationic lipids such as 3β[N-N',N'-dimethylamino ethane-carbamoyl] cholesterol (DC-cholesterol) mixed in varying molar ratios with the neutral phospholipid, dioleoylphosphatidylethanolamine (DOPE) (Caplen *et al*, 1995). Detergents and cholesterol, as in DC-cholesterol may be added to increase stability. DC-cholesterol has two disadvantages; it is unstable with a short shelf life of only five weeks and it is not readily available in bulk.

The negatively charged DNA component from a bacterial plasmid containing the CFTR cDNA with a strong heterologous promotor combines to the cationic liposome forming a liposome/gene complex. The mechanism by which the complexes mediate gene transfer is unknown but they may enter the cell by either fusing to the cell membrane or via endocytosis. The latter system is thought to be the most likely. Once inside the cell the plasmid DNA most likely remains episomal (Sorscher *et al*, 1994).

Cationic liposomes are associated with low toxicity which makes them very attractive as a gene therapy vector. They have the ability to transfect a variety of cell types, are easy to prepare and can accomodate and deliver genes of almost unlimited

size. However, cationic liposomes appear to be a less effective delivery system than adenoviruses but the latter vector has been associated with an inflammatory response in CF patients.

Another potential vector are adeno-associated viruses (AAV). These are small single-stranded DNA parvoviruses that are nonpathogenic. They are generally unable to replicate autonomously and require a helper virus such as adenovirus or Herpes simplex. When an AAV infects a cell in the absence of a helper virus it integrates into the host cell genome and becomes latent. The AAV genome is only 4.7kb and therefore most of the genome has to be removed to accomodate the CFTR cDNA which may lead to inefficient packaging and difficulties in virus production. However, *in vitro* studies have shown that AAV vectors are capable of correcting the CF Cl^- permeability defect (Flotte *et al*, 1993) and a clinical trial is ongoing (Rosenfeld & Collins, 1996). Although AAV vectors have shown promise a number of disadvantages have become apparent; those include difficulty in obtaining large quantities of the virus in high enough titres. Furthermore, *in vivo* studies, in monkeys, have shown that the AAV vector may not always remain at the site of administration following airway delivery (Flotte *et al*, 1995).

1.7.2 Gene therapy trials

A number of gene therapy trials in CF adults have been completed or are currently underway in the USA, UK and France. The majority of these are Phase I ('proof of principle') trials which involve local application of the adenovirus-mediated gene or

the liposome/gene complex to the nasal mucosa. The principal objective of the Phase I trials is to establish the efficacy of the delivery system and to determine whether there are any adverse reactions to the vectors used to deliver the gene.

The vector of choice, in the USA, is the modified adenovirus and the first Phase I clinical trial showed encouraging results with regard to functional correction although only three patients were involved (Zabner *et al*, 1993). The first application of gene therapy to the upper and lower airways of four CF patients, again using adenovirus-mediated gene delivery, demonstrated that gene transfer was only successful in one of the patients. Furthermore, a severe inflammatory reaction occured in another patient emphasing the potential problems associated with adenovirus vectors (Crystal *et al*, 1994). A further study, in 12 patients, using escalating doses of the adenovirus-mediated gene complex to the nasal mucosa showed no evidence of gene transfer at the lower doses but CFTR was expressed in about 1% of the cells, at higher doses. Evidence of mucosal inflammation was demonstrated at the highest dose of vector and no functional correction was observed (Knowles *et al*, 1995).

The first clinical trial of DNA-liposome gene therapy for CF, using DC-cholesterol (DOPE) administration to the nasal epithelium demonstrated transgene delivery and expression by biopsy and showed around 20% correction of the electrophysiological deficit 3 days after therapy; this affect disappeared by day 7 (Caplen *et al*, 1995).

Unfortunately, false positive results in the placebo group limited the interpretation of evidence of gene transfer in this trial.

The Edinburgh Phase I gene therapy trial, used an alternative cationic liposome, DOTAP (N,N,N-trimethyl-2-3-bis ((1-oxo-9-octa-decenyl)oxy)-(Z,Z)-1-propanaminium methyl sulphate) which was produced by Boehringer Mannheim, Germany (Porteous *et al*, 1997). This liposome was chosen for the human trial as repeat administrations of the liposome to the Edinburgh transgenic CF mutant mouse were tolerated well (McLachlan *et al*, 1995) and when complexed with CFTR using cytomegalovirus (CMV) as a promotor resulted in approximately 80% correction of tracheal electrophysiological deficit in these animals (McLachlan *et al*, 1996). The CMV promotor was chosen as studies in mice showed that it sustained expression for at least 17 days in comparison to simian virus 40 (SM40) which was attenuated rapidly (Caplen *et al*, 1995; McLachlan *et al*, 1995).

The first Edinburgh gene therapy trial recruited 23 patients but only 16 of these individuals completed the trial. Eight patients were treated with the pCMV-CFTR-DOTAP while the other eight patients received a placebo of buffer only. Evidence of gene transfer was detected in seven of the eight treated patients at days three and seven, post therapy, but in only two of the seven patients at 28 days. A partial correction of the chloride abnormality averaging 20 % was detected, which is similar to a previous study by Caplen *et al* (1995); in the Edinburgh trial no false positive results were observed.

For ethical reasons all CF human gene therapy trials to date have been carried out in adult CF patients. However, this group of patients are unlikely to benefit significantly as their lungs are already badly scarred due to recurrent pulmonary infections and inflammatory damage. Ideally, CF patients should receive gene therapy prior to the acquisition of *P. aeruginosa* as gene therapy only stops rather than repairs progressive lung damage and the inflammatory obstructions and microbial alginate produced by *P. aeruginosa* hinder the nebulised gene therapy from reaching the target cells in the respiratory tract.

CHAPTER 2

EPIDEMIOLOGY OF CF PULMONARY INFECTIONS

"In clinical practice, bacterial typing systems are applied primarily to address one fundamental issue: are two isolates the 'same' or 'different' ?" (Maslow *et al*, 1993).

Epidemiological studies can provide insight into the spread and persistance of pathogens, determine clonally related isolates of a species or provide evidence when reinfection occurs as to whether this is due to the original isolate or acquisition of a new strain. Epidemiological investigations have utilised a wide variety of bacterial typing systems which can be conveniently classified into two categories: phenotypic systems are based on detection of characteristics expressed by microorganisms; genomic systems involve direct DNA-based analyses of chromosomal or extra-chromosomal genetic elements. A list of phenotypic and genomic typing systems together with their advantages and disadvantages are shown in Table 2.1.

An ideal typing system should possess the properties of reproducibility, stability, discrimination and typeability (i.e. be able to assign a type to a high percentage of isolates). Additional advantages would include rapid, inexpensive and technically simple procedures with application to a wide range of different species.

TABLE 2.1. Phenotypic and genomic typing systems

Phenotypic typing methods

Biotyping
Serotyping
Antimicrobial susceptibility
Bacteriophage typing
Bacteriocin typing
Protein analysis
Multilocus enzyme electrophoresis

Advantages	Disadvantages
Simple to perform	poor reproducibility
rapid	insufficient discrimination
inexpensive	limited applicability
requires no specialised equipment	unstable

Genomic typing methods

Plasmid profile analysis
Restriction endonuclease analysis (REA)
Ribotyping
Pulsed-field gel electrophoresis
PCR techniques

Advantages	Disadvantages
reproducible	requires technical expertise
discriminatory	time consuming
stable	requires specialised, expensive
broad applicability	equipment

2.1 PHENOTYPIC TYPING SYSTEMS

Phenotypic typing systems (biotyping, serotyping, phage typing etc.) include classic techniques for differentiating strains and are still commonly used in clinical laboratories, although they are increasingly being superceded by genomic methods. One of the main disadvantages of phenotypic typing systems is that organisms are

prone to phenotypic variation resulting in the possibility of clonal isolates being classified as different types. None of the phenotypic typing systems offer an ideal approach to subdividing a microbial species as there are problems related to typeability, reproduciblity or discriminatory powers. Furthermore, phenotypic typing systems are not generally applicable. Instead, they have to be developed for a particular organism and are not easily applied to fastidious organisms.

Unlike the other simple phenotypic typing systems, typing by protein analysis and multilocus enzyme electrophoresis (MLEE) involve more complex techniques. They are also time consuming and require specialised equipment.

2.1.1 Biotyping

Biotyping is based on the discriminatory results of a range of biochemical tests to provide a biochemical profile from which different biotypes can be identified. In most clinical laboratories conventional biochemical tests for identification of organisms have largely been replaced by automated systems, such as API and Vitek systems. Biotyping is not a reliable typing technique as variation in gene expression may alter biochemical reactions leading to the production of different biotyping patterns in otherwise clonally-related strains. In general, the discriminatory powers of biotyping are poor which is probably not surprising considering that biotyping systems for some organisms only involves a few biochemical tests. Biotyping has been retained and used for epidemiological investigations in some bacteria, notably for *H. influenzae*.

2.1.2 Antimicrobial susceptibility

Although this technique is performed routinely in clinical laboratories it is of limited use in epidemiological studies because of phenotypic variation and the emergence of antibiotic resistance by selective pressure. Resistance can rapidly evolve within a strain through various genetic mechanisms or by means of resistance plasmids; both mechanisms are potentially unstable resulting in altered antibiogram patterns. Consequently, two isolates of the same strain may exhibit different antibiogram patterns and conversely, different strains may demonstrate similar antibiogram patterns.

2.1.3 Serotyping

Serotyping is one of the oldest typing systems and is largely used for epidemiological studies of Gram negative bacteria such as *Escherichia coli*, *H. influenzae*, *P. aeruginosa*, *Neisseria meningitidis* and some Gram positive bacteria such as *Streptococcus pneumoniae* (Maslow *et al*, 1993). This immunological technique detects expression of distinct antigenic determinants on the cell surface. Specific typing reagents are required such as polyclonal sera and monoclonal antibodies which are often difficult and expensive to develop, and in some cases are not readily available. Serotyping can be applied to many different bacterial pathogens but in most cases a given set of antisera can only be applied to a single species. This technique is relatively stable and reliable but often demonstrates poor discriminatory powers. In CF patients, loss of LPS side chains in *P. aeruginosa* also results in an

unacceptable number of untypeable or polyagglutinating isolates (Hancock *et al*, 1983).

2.1.4 Bacteriophage typing

A number of lytic bacteriophages or viruses capable of infecting bacterial cells, have been identified. Isolates are characterised according to their susceptibility or resistance to lysis by a panel of bacteriophages for a particular species. Phage typing schemes have been developed for numerous bacterial genera but is notably the major typing method for *Staph. aureus* (Blair & Williams, 1961). This technique is generally restricted to reference laboratories due to the maintenance of stocks of active typing phages. Bacteriophage typing is highly sensitive and is subject to considerable experimental and environmental variability. Genetic mechanisms such as lysogenic conversion, loss of prophages, LPS variation and the acquisition or loss of plasmids can affect the phage type.

2.1.5 Bacteriocin typing

The technique for typing isolates of *P. aeruginosa* was developed in this laboratory in 1966 by Gillies & Govan and was later modified by Fyfe *et al* in 1984. A similar technique was developed for *B. cepacia* in 1986 by Govan & Harris. Many bacterial species produce bacteriocins, a heterogeneous group of antimicrobial peptides and defective phage particles are active against different strains of the same species with the producing strain generally being resistant or immune. Bacteriocin typing is based on the pattern of inhibition exhibited by the production of bacteriocins by the test

strain against a set of indicator strains, or alternatively, susceptibility of a test strain to bacteriocins produced by standard producer strains. The inhibition patterns determine the bacteriocin type of the organism. This technique requires considerable developmental work but has been applied successfully to *P. aeruginosa*, *B. cepacia* and *Enterobacteriacae*. It is a relatively discriminating typing system but like other phenotypic systems, lacks reproducibility. Although production of bacteriocins or the susceptibility to them are relatively stable properties, the presence of transferrable R plasmids may lead to a change in bacteriocin type.

2.1.6 Protein analysis

Variations in the structure of bacterial proteins can be detected by a variety of methods. The solubilised material isolated from whole-cell or cell-surface proteins is separated by electrophoresis and detected by staining techniques to generate a characteristic protein profile. One of the most widely used techniques is SDS-PAGE (sodium dodecylsulphate polyacrylamide gel electrophoresis). Proteins are denatured by heat, detergent and reducing agents to produce polypeptide subunits which are separated by electrophoresis on the basis of molecular size. Alternatively, proteins can be radiolabelled during isolation and the pattern detected by autoradiography. It is important to standardise growth conditions such as media, inoculum size, incubation conditions (time, temperature, gaseous atmosphere) for harvesting and washing cells as the protein content of microbial cells may be influenced by environmental factors. Virtually all bacteria are typeable by this method. However,

interpretation of results may prove difficult due to the large number of bands produced in the electrophoretic patterns.

2.1.7 Multilocus enzyme electrophoresis (MLEE)

In MLEE, microorganisms are differentiated by analysis of electrophoretic mobility of a range of soluble basic metabolic enzymes extracted from the organism. The cellular proteins of the microorganism are separated by starch gel electrophoresis and individual soluble metabolic enzymes are detected using specific subtrates and chromogens. MLEE is technically demanding and is generally only moderately discriminatory for clinical isolates. Consequently, this typing method has had relatively limited application to epidemiological studies. However, the discrimination of isolates and typeability by this technique may be enhanced by analysis of multiple enzymes and a panel of 10 - 15 is commonly used. Combined data for the range of enzymes tested is then used to assign an electrophoretotype to a particular isolate which allows comparison with other isolates. Single amino acid changes in enzymatic proteins encoded by different alleles or genes are sufficient to change the electrophoretotype. As with protein analysis, the amount of enzyme produced may be affected by cultural and environmental conditions.

2.2 GENOMIC TYPING SYSTEMS

The limitations inherent to traditional methods of typing microorganisms by phenotypic characteristics stimulated the development of techniques relying on the detection of polymorphisms at the level of nucleic acids (genotyping). Recent

advances in nucleic acid technology have resulted in the emergence of a number of genotypic typing methods. Initially, these methods were only used in research laboratories due to the complexity of the techniques, complex interpretation of results and the expensive equipment required. However, many of the techniques are now being used in clinical laboratories. A particular genotyping system can be applied to a wide range of bacteria in comparison to phenotypic methods which generally have to be developed for each species of organism. However, genotyping systems are not without their own particular drawbacks such as being time consuming and requiring considerable technical expertise both in performing and the interpretation of results.

2.2.1 Plasmid profile analysis

Plasmid analysis was one of the first DNA-based techniques to be applied to epidemiological studies of bacterial pathogens (Meyers *et al*, 1976). Basically, plasmid DNA is extracted from an isolate and separated by electrophoresis in an agarose gel to determine the molecular size and number of plasmids present. Additional information can be obtained by digestion of the plasmid DNA, with restriction enzymes, prior to separation by electrophoresis. The agarose gels are then stained with ethidium bromide and the plasmid profiles then observed by exposure to UV light. Plasmid analysis is technically the simplest of the DNA-based methods and requires only basic electrophoresis equipment. A major drawback of this typing system is that plasmids are not always present. Even when they are present, stability can prove a problem with plasmids being spontaneously lost or acquired.

2.2.2 Restriction endonuclease analysis of chromosomal DNA (REA)

DNA is enzymatically cut or digested with restriction endonucleases or enzymes at a particular 'restricted' nucleotide recognition sequence (Maslow *et al*, 1993; Kerr, 1994). The number and size of the restriction fragments generated by digestion of the particular piece of DNA reflect the frequency and distribution of such restriction sites. Restriction enzymes with relatively frequent restriction sites are used to digest bacterial DNA, resulting in hundreds of fragments, ranging from ~ 0.5kb to 50kb in length, which are subsequently separated by agarose gel electrophoresis. The agarose gels are stained with ethidium bromide and the banding patterns examined under UV light.

REA has been successfully applied to many species of bacteria (Towner & Cockayne, 1993) and the majority of isolates found to be typeable. Different strains of the same species have different REA profiles because of variations in their DNA sequences. The major limitation of this method is the difficulty in interpretation of the DNA fragment profiles due to the large number of closely spaced bands produced (Maslow *et al*, 1993). The presence of plasmids may also alter REA profiles resulting in isolates that differ only in their plasmid content to be designated as different strains. Variation in the number and size of fragments, due to sequence re-arrangments, insertions and deletions of DNA within a particular restriction site, produced by REA are referred to as restriction fragment length polymorphisms (RFLPs).

2.2.3 Southern blotting

Southern blotting, named after the investigator who first descibed this procedure (Southern, 1975), reduces the number of observed DNA fragments produced by REA. In Southern blotting, the separated restriction fragments are transferred to a nitrocellulose or nylon membrane and a labeled fragment of DNA as a probe can detect the restriction fragment(s) which hybridise with sequences homologous to the probe. All strains carrying sequences homologous to the probe are typeable and results are highly reproducible. The procedure requires technical expertise and originally utilised radioisotopes to label the probe. Nonradioactive detective systems for Southern blotting are now available. The discriminatory power of this technique is related to the number and variability of the fragments detected.

2.2.4 Ribotyping

Ribotyping, first described by Grimont & Grimont (1986), is the analysis of DNA polymorphisms in the chromosomal regions containing highly conserved rRNA genes. The rRNA genes are organised into operons, within which the individual genes coding for 16S, 23S and 5S RNA are often separated by non-coding DNA spacer regions. Ribotyping involves Southern blotting analysis using a rRNA probe for typing purposes. In many bacteria, considerable variation occurs within the 16S-23S spacer region, both in length and sequence. These variations are detected, following hybridisation with a probe specific to this region, by the resulting pattern of DNA fragments produced. Isolates are typed according to the different banding patterns produced which are refered to as ribotypes.

Various probes can be used for ribotyping including labelled mixtures of 16S and 23S RNA resulting in hybridisation only with the fragments of the chromosomal DNA which contain parts of the corresponding gene. Commercially available rRNA probes, derived from *E. coli* ribosomal operons, can be used as 'universal' probes to ribotype a wide range of bacteria. However, specialised probes may give better specificity and discrimination within a species.

Many organisms carry multiple (5 - 7) ribosomal operons including *E. coli*, *Staph. aureus* and *H. influenzae.* Thus the ribotype patterns produced comprising 10 - 15 bands provide moderate to good discrimination. In contrast ribotyping is of limited use in mycobacteria which have only a single operon and produce only 1 - 2 ribotype bands (Maslow *et al*, 1993). Ribosomal genes are relatively stable because they are located on the chromosome, however, epidemiologically unrelated isolates sometimes demonstrate the same ribotype pattern limiting the discriminatory power and reliability of this typing system. Other factors affecting the level of ribotype discrimination include the precision of the probe and restriction enzyme(s) used.

Ribotyping is reproducible but is time-consuming and relatively complex to perform. An advantage is that interpretation of the results is relatively simple. Ribotyping has the disadvantages that are associated with the use of radioactive material including exposure hazards, short half-lives and disposal of waste material. However, non-isotopic methods are available such as biotinylated probes (Pitcher *et al*, 1987) and chemiluminescence (Gustaferro & Persing, 1992) to examine rRNA restriction

patterns. The main discriminatory disadvantage of ribotyping is that it only examines the specific region of the genome which hybridises with the particular probe used. This contrasts with other genomic typing systems whose discriminatory potential benefits from examination of the entire bacterial chromosome.

2.2.5 Polymerase chain reaction (PCR)

PCR is an enzymic method for exponentially amplifying specific nucleic acid sequences of DNA and was introduced by Saiki *et al* in 1985. This technique has a wide variety of applications including detection, identification and epidemiological typing of miroorganisms. Amplification of specific sequences is achieved with a DNA template, oligonucleotide primers, deoxynucleotide triphosphates (dNTP's) and a thermostable DNA polmerase in a suitable buffer. Each PCR amplification is sub-divided into three steps comprising denaturation, primer annealing and primer extension, which are repeated in cycles. The double stranded DNA is denatured by heating to over 90^{o}C, resulting in separation of the strands. The sample is then cooled to around 50^{o}C to allow the primer(s) to hybridise to their complementary sequences and thereby initiate copying of the single stranded template. The final process involves an extension of the annealed primers by polymerase-mediated nucleotide additions to produce two copies of the original sequence. This is achieved by heating to 70 - 75^{o}C, which is the optimum temperature for heat-stable DNA polymerases. Once this cycle is completed, the sample is heated again to enable a further round of DNA amplification to take place.

The original method for PCR used DNA polymerase I (Klenow fragment) from *E. coli* (Saiki *et al*, 1988). However, the high temperatures necessary for denaturation of DNA inactivated this enzyme resulting in the need to replenish the enzyme before every cycle after restoration of the sample temperature to 37^{o}C. This enzyme was subsequently replaced by a thermostable DNA polymerase (*Taq*) isolated from *Thermus aquaticus* (Saiki *et al*, 1988) which can withstand repeated exposure to the high temperatures of 94 - 95 ^{o}C. *T. aquaticus* is a thermophilic eubacterium found in hot springs and water heaters. Another benefit achieved by using *Taq* DNA polymerase is that it can withstand the rapid 37^{o} to 96^{o}C temperature changes necessary for PCR amplication.

A number of factors are important for a successful PCR reaction to occur, the most important being the selection of primer. The primer should have a GC content similar to the fragment being amplified. In addition, the concentration of $MgCl_2$ in the buffer can have a profound effect on the specificity and yield of an amplification. Generally, excess Mg^{++} will result in accumulation of non-specific amplification products and insufficient Mg^{++} will reduce the yield. dNTP's appear to quantitatively bind Mg^{++} therefore the amount of dNTP's present in a reaction will determine the amount of free Mg^{++} available. Temperature, Mg^{++} concentration and *Taq* polymerase concentration are all known to exert a strong effect on the specificity of a PCR reaction. Annealing temperatures depend on the length and GC content of the primers. PCR is an extremely powerful technique and a common problem is

cross-contamination: PCR is so sensitive that it has been said that it is capable of detecting a single molecule in a swimming pool.

A number of PCR-based typing techniques have been developed including PCR-ribotyping, arbitrarily-primed PCR (AP-PCR) or random amplification of polymorphic DNA (RAPD-PCR), and enterobacterial repetitive intergenic consensus (ERIC-PCR).

2.2.6 PCR-ribotyping

This technique is an alternative approach to traditional ribotyping, uses PCR to amplify the 16S-23S intergenic spacer region of the bacterial rRNA operon for the specific purpose of detecting heterogeneity between and within species (Kostman *et al*, 1992). The choice of primers is critical for successful detection of spacer variation. PCR- ribotyping detects variation in length polymorphism and sequence heterogeneity within the intergenic spacer regions by PCR amplification using specific oligonucleotide primers (Kostman *et al*, 1992). These primers are complementary to the highly conserved regions flanking the intergenic spacers of the ribosomal RNA genes. The resultant PCR product is then separated by agarose gel electrophoreisis. The epidemiological relatedness of microorganisms can be determined by comparison of the amplification patterns produced by PCR-ribotyping. As only a small number of bands are produced, greater discrimination can be achieved by further digestion of the PCR product, using restriction endonucleases, prior to separation by electrophoresis. Primer specificity and high

annealing temperatures make random amplification of the bacterial genome unlikely in PCR-ribotyping. PCR-ribotyping is a rapid, reproducible, stable and an adaptable method for molecular epidemiologic analysis. As most bacteria contain multiple copies of ribosomal RNA genes, this technique can be applied to type many different genera and species of bacteria. However, as mentioned in the introduction to conventional ribotyping, certain bacterial species have only one or two rRNA operons per genome and subsequently the spacer length and variation among such species is limited.

2.2.7 AP-PCR or RAPD

Conventional PCR techniques amplify DNA sequences that are characteristic of a particular species of bacteria, using specific oligonucleotide primers. A prior knowledge of the DNA sequence of the organism under investigation is therefore a prerequisite. This requirement does not apply to RAPD as amplification of template DNA is achieved by a single arbitrary primer which is not complementary to any designated target but instead relies on the primer binding at multiple sites on the bacterial chromosome. RAPD is carried out under conditions of low stringency (low annealing temperatures), in the early cycles, followed by numerous cycles at high stringency resulting in the primers annealing sufficiently close together in the correct orientation with subsequent amplification of the intervening sequences. RAPD typing is based on polymorphic variations in the intensity, number and length of the multiple amplification products. RAPD analysis is rapid and cost effective but its reproducibility is a subject of debate. Factors which affect reproducibility are the

extraction methods employed for DNA (Gomez-Lus *et al*, 1992), concentration of template DNA (Davin-Repli *et al*, 1995) and selection of primers, polymerase and cycling conditions (Kerr, 1994). Identifying a suitable primer that provides consistent, reproducible results may be difficult.

2.2.8 ERIC-PCR

Repetitive DNA sequences have been identified in bacterial genomes and are widely distributed throughout the chromosome. ERIC sequences are ~126bp and occur singly at each location on the chromosome. Primers based on ERIC sequences are used for PCR amplification of genomic DNA and the resulting PCR product is separated by agarose gel electrophoresis. The banding patterns produced by ERIC-PCR represent amplification of DNA between adjacant repetitive elements within the limits of polymerase extension and can be used to fingerprint bacteria. Differences in band sizes probably result from polymorphisms in the distance between ERIC sequences in different genomes. An advantage of ERIC-PCR typing is that DNA does not have to be extracted as whole bacterial cells can be used.

2.3 PULSED FIELD GEL ELECTROPHORESIS

"It is unquestionable that PFGE is the 'golden standard' for typing most bacterial species" (Liu *et al*, 1995).

For the purposes of genomic fingerprinting, the problem of complex REA banding patterns resolved to some extent by the use of rare cutting restriction enzymes which

digest chromosomal DNA at infrequent occuring restriction sites yielding 5 - 30 fragments of much larger sizes ranging from ~10kb to 800kb in length. In terms of DNA analysis, conventional gel electrophoresis is dependant on the migration of DNA molecules through a solid matrix, usually agarose, under a static electric field. This is a disadvantage as it can only separate molecules less than 50kb. In contrast, PFGE can separate much larger DNA fragments up to 7Mb. During conventional gel electrophoresis, the DNA molecules elongate, align with the electric field and migrate towards the anode. Attempts were made to extend the range of separation by reducing the concentration of agarose and the use of very low voltage gradients but this resulted in gels which were difficult to handle and run times of several days or weeks only to achieve separation of at the most a few hundred kilobase pairs.

Klotz and Zimm (1972) demonstrated that DNA molecules elongate in the direction of the electric field. When this field is withdrawn the DNA molecules relax back to their original state, with the rate of relaxation dependant on the length of DNA. Schwartz, a student of Zimm, focused on this size-dependant relaxation and succeeded in separating large DNA molecules. He found that changing the orientation of the electric field forced the DNA molecules in the gel to relax on removal of the first electric field, then elongate and align with the new field. Schwartz *et al* (1982) introduced pulsed-field gel electophoresis (PFGE) as a method of separating DNA molecules larger than 50kb by using two alternating electric fields, one homogeneous and the other non-homogeneous. Their first study was applied to yeast chromosomes to demonstrate the effectiveness of field switching or

pulsing to separate high molecular weight DNA molecules in the range of 200-3000kb. PFGE works on the principle that DNA molecules, in the agarose gel, elongate in the direction of the electric field and begin to migrate through the gel. When the first electric field is removed, and a second field at an angle to the first is activated, the DNA molecules change conformation and reorientate before they can migrate in the direction of the second electric field. The time required for reorientation is dependant on the length of the DNA molecule, with larger molecules taking longer to realign than smaller ones due to the physical barrier of the agarose matrix. Provided the alternating electric fields are equal in length and voltage, the DNA will migrate in a straight path through the gel.

A number of different 'commercial' PFGE systems are now available. Each has the ability to separate the same range of DNA sizes but differs in the speed of separation and the resolution obtained in any specific size range. In a variation to PFGE, Chu *et al* (1986) applied the principles of electrostatics to generate homogeneous electric fields using multiple electrodes arranged around a closed contour, known as contour clamped homogeneous electric field electrophoresis (CHEF). The 24 electrodes are arranged in a hexagonal configuration with the voltage divided between these electrodes so that the voltage gradient produced is constant across the gel. The CHEF DRII system has a fixed angle between the direction of the pulsed fields (reorientation angle) of 120^{o}C but the newer model CHEF DRIII has a variable reorientation angle of 90 - 120^{o}, which can speed up the separation of the very large molecules. The major advantage of the CHEF system is its ability to separate a large

number of DNA samples in straight lanes due to the use of homogeneous electric fields. Chu *et al* (1986) also used yeast chromosomes to demonstrate that homogeneous electric fields are necessary to separate large DNA molecules in straight lanes in pulsed-field gels.

2.3.1 Preparation of DNA for PFGE

Bacteria are embedded in low melting agarose plugs prior to the lysing process. The agarose allows free flow of solutions necessary for lysing the bacteria and subsequent digestion of the chromosomal DNA. Materials released during the lysis and digestion procedures diffuse out of the agarose during washing, while the DNA remains intact. The agarose protects the long strands of DNA from mechanical breakdown and nucleolytic degradation. DNA can remain intact, without degeneration, when stored in an appropriate buffer for several years.

The optimisation of a number of variable parameters is necessary to obtain good separation of DNA fragments by PFGE. These variables include: pulse times, voltage gradient, type and concentration of agarose, buffer composition, temperature, and run time.

2.3.2 Pulse times

This is the most crucial factor in obtaining good separation of chromosomal DNA. The size range of fragments resolved is directly proportional to the pulse time, which can vary from a fraction of a second, which will separate molecules of a few

kilobases to over an hour for molecules >5Mb. Ramping procedures are generally used to separate a range of different sized DNA fragments starting with a short initial pulse time which gradually increases during the run time until the longer final pulse time is reached. The initial and final pulse times are dependant on the size range of the fragments to be resolved.

2.3.3 Voltage gradient

This is the difference between the electrical potential of the electrodes in the gel tank, and represents the force that drives the DNA molecules through the gel. Different voltage gradients are used for separating different sizes of DNA, fragments with larger DNA molecules requiring lower voltage.

2.3.4 Type and concentration of agarose

Low melting agarose has reduced gel strength and smaller pore size than regular agarose gels and is used for the preparation of agarose plugs, as already mentioned. Pulsed-field gel agarose has to have a high degree of purity. It also has a precise level of charged constituents present. Since the DNA migrates faster as the level of charge is reduced.

Unlike conventional electrophoresis, where different concentrations of agarose are used to separate different size ranges of DNA fragments, changing the concentration of agarose of PF gels affects the speed at which the DNA migrates through the gel.

As the agarose concentration decreases the DNA migrates faster but the bands appear less sharp.

2.3.5 Composition of running buffer

The composition of the buffer affects the mobility and resolution of DNA through the gel. DNA migrates quicker in buffers of low ionic strength. The two main buffers used for PFGE are Tris-borate-EDTA (TBE) and Tris-acetate-EDTA (TAE). DNA fragments run faster in TAE buffer and this is generally used for the separation of larger fragments but the buffer has to be changed frequently to maintain its buffering capacity. TBE buffer is used to separate fragments <2Mb and has a greater buffering capacity than TAE buffer.

2.3.6 Temperature of running buffer

As the temperature of the buffer increases, the DNA fragments migrate more quickly; however, the resolution diminishes. The migration of DNA fragments is sensitive to change in temperature therefore it is important to maintain the running buffer at a constant temperature to ensure equal migration in each of the lanes.

2.3.7 Run time

In PFGE, the run time of the gel depends on the range of sizes of DNA fragments to be resolved and the pulse times used. The temperature of the running buffer also affects the run time as the cooler the temperature the longer the run time. Larger gels

require longer run times but the further the bands migrate down the gel the less sharp they become.

PFGE has been described as being nearly the optimal typing method, certainly for isolates of *E. coli* and *Mycobacterium avium* (Maslow *et al*, 1993). This technique provides a highly reproducible restriction profile that typically shows distinct well-resolved fragments representing the entire bacterial chromosome in a single gel. Several studies have documented the superiority of PFGE in comparison to a variety of phenotypic and other molecular epidemiological typing methods (Goering *et al*, 1990; Saulnier *et al*,1993). PFGE also has been referred to as the gold standard for epidemiological analysis of nosocomial infection (Goering, 1993). PFGE demonstrates excellent reproducibility and discrimination (Anderson *et al*, 1991; Bingen *et al*, 1993; Steinbach *et al*, 1994) and can be applied to virtually any organism from which the DNA can be extracted. Within the past few years, PFGE technology has been applied to the epidemiological analysis of a large number of microorganisms, as shown in Table 2.2. As with all typing systems, there are a number of disadvantages; these include the understood fact that PFGE is time consuming, technically demanding and requires the use of expensive equipment.

Table 2.2. Examples of microorganisms investigated by PFGE of chromosomal DNA fragments

Organism	Restriction enzyme	Reference
Acinetobacter baumannii	*Apa*I	Gouby *et al* (1992)
Bacillus cereus	*Xba*I	Liu *et al* (1997)
Bordetella pertusis	*Xba*I	Syedabubakar*et al* (1995)
Burkholderia cepacia	*Spe*I	Anderson *et al* (1991)
Burkholderia picketti	*Spe*I	Chetoui *et al* (1997)
Campylobacter jejuni	*Sma*I	Yan *et al* (1991)
Campylobacter fetus	*Sma*I	Fujita *et al* (1995)
Clostridium difficle	*Sma*I	Kristjansson *et al* (1994)
Corynebacterium diphtheriae	*Sfi*I	Dezoysa *et al* (1995)
Enterobacter cloacae	*Not*I/*Xba*I	Haertl & Bandlow (1993)
Enterobacter faecium	*Sma*I	Miranda *et al* (1991)
Enterococcus faecalis	*Sma*I	Tomayko & Murray (1995)
Escherichia coli	*Xba*I	Bohm & Karch (1992)
Klebsiella pneumoniae	*Xba*I	Gori *et al* (1996)
Legionella pneumophilia	*Bss*HII/*Sal*I/*Spe*I	Johnson *et al* (1994)
Listeria monocytogenes	*Apa*I/*Sma*I	Brosch *et al* (1991)
Mycobacterium avium	*Ssp*I	Coffin *et al* (1992)
Mycobacterium fortuitum	*Xba*I	Hector *et al* (1992)
Mycobacteria tuberculosis	*Xba*I/*Spe*I	Zhang *et al* (1992)
Neisseria gonorrhoeae	*Xba*I/*Nhe*I	Xia *et al.* (1995)
Neisseria meningitidis	*Sfi*I/*Spe*I/*Nhe*I	Bygraves & Maiden (1992)
Pseudomonas aeruginosa	*Xba*I/*Dra*I	Bennekov *et al* (1996)
Shigella sonnei	*Xba*I	Litwin *et al* (1997)
Staphylococcus aureus	*Sma*I	Bannerman *et al* (1995)
Staphylococcus epidermidis	*Sma*I	Hu *et al.* (1995)
Streptococcus thermophilus	*Sma*I/*Apa*I/*Sfi*I	Boutrou *et al.* (1995)
Salmonella typhi	*Xba*I/*Spe*I	Nair *et al.* (1994)
Salmonella enteritidis	*Xba*I/*Spe*I/*Avr*II	Thong *et al.* (1995)
Vibrio cholera	*Not*I / *Sfi*I	Mahalingam *et al.* (1994)
Yersinia enterocolitica	*Not*I	Najdenski *et al.* (1994)

2.3.8 Interpretation of banding patterns

The interpretation of the banding patterns produced by PFGE can prove difficult, especially in determining the relatedness of a number of isolates. Alterations may occur in both the number and position of the bands due to the presence of plasmids or bacteriophage DNA (Towner & Cockayne, 1993). A single band difference may be due to genetic variation. Point mutations resulting in gain or loss of a restriction site will result in a three band difference. Insertion or deletion of DNA into or from a fragment will result in a two band difference. It is now generally accepted that isolates demonstrating up to a three band difference in their PFGE profiles are regarded as being closely related (Tenover *et al*, 1995) or subtypes of the same strain (Goering, 1993). A 4 - 6 band difference demonstrates strains which are possibly related (Tenover *et al*, 1995), as two genetic events could result in up to a six band difference.

PFGE banding variations have been observed among isolates collected over long periods of time. However, isolates that have no epidemiological link are likely to be different if they demonstrate more than a three band difference. Isolates producing more than a six band difference are classified as different strains.

The Dice coefficient (Dice, 1945) can be used to determine the degree of similarity in the banding patterns of two isolates using the following formula (Steinbach *et al*, 1994):

Dice coefficient of similarity (D)

$D = 2n_{xy} / n_1 + n_2$ where n_1 = total number of DNA fragments from strain x

n_2 = total number of DNA fragments from strain y

n_{xy} = number of identical fragments

A D value of > 0.9 represents closely related strains

< 0.6 unrelated strains

As PFGE is considered to be the 'gold standard' typing method for fingerprinting isolates of microorganisms, it was proposed to investigate the use of this system for typing CF pathogens in this thesis.

CHAPTER 3

GENERAL AIMS

The general aims of this thesis focus on the major bacterial pathogens responsible for pulmonary infection in CF patients. As mentioned previously, the spectrum of pathogens is limited and primarily comprises; *Staphylococcus aureus, Haemophilus influenzae, Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Stenotrophomonas maltophilia* has also been investigated although the pathogenic role of this organism in CF patients remains unclear. The thesis investigates the major epidemiological issues of each pathogen focusing, in particular, on the application of the genomic typing system, pulsed-field gel electrophoresis. More precise aims will be addressed in the chapter covering the individual pathogen.

3.1 *STAPHYLOCOCCUS AUREUS*

Staph. aureus is a common commensal of the anterior nares in many healthy individuals with a higher incidence in infants and young children than in adults (Gould & McKillop, 1954). As this organism is generally the first pathogen to cause pulmonary infections in CF patients, particularly in babies and young children, it has been speculated that nasal *Staph. aureus* acts as a reservoir which subsequently leads to colonisation of the lower respiratory tract and pulmonary infection. Nasal flora has been shown to be responsible for endogenous infections in other groups of patients (White, 1963). The aim of this section of the thesis is to examine isolates of

Staph. aureus cultured from nasal and sputum specimens from the same CF patient by PFGE to confirm or reject the hypothesis that nasal carriage of *Staph. aureus* is responsible for subsequent pulmonary infection in CF patients.

Pulmonary infection due to *Staph. aureus* is less common in adult CF patients than in paediatric CF patients. Could this be attributed to a lower nasal carriage rate of *Staph. aureus*, assuming that the carriage rate in CF patients is the same as for healthy individuals? To investigate this hypothesis, nasal swabs from paediatric and adult CF patients were investigated to determine the incidence of nasal carriage of *Staph. aureus* in paediatric and adult CF patients and in healthy individuals.

Sixteen CF patients were involved in a Phase I gene therapy trial at the Western General Hospital, Edinburgh. This was basically a safety trial to show that a single local application of the liposome/gene complex to the nasal epithelial cells had no adverse affects on the patient. The liposome/gene complex was administered to eight patients and the remaining eight patients received a placebo. Sputum and swabs of the anterior nares were taken at various intervals throughout the trial to monitor the effect of treatment on the bacterial flora, with particular interest in *Staph. aureus*. Subsequently, nasal swabs were obtained from this group of patients to investigate the length of time that a strain of *Staph. aureus* colonises the nose of an individual patient.

3.2 *HAEMOPHILUS INFLUENZAE*

H. influenzae is present in the majority of healthy individuals as an upper respiratory tract commensal. However, *H. influenzae* is a common cause of pulmonary infection in CF patients, especially in infants and young children. Consequently, the significance of *H. influenzae* recovered from respiratory secretion of CF patients poses a problem i.e. in determining whether the organism is merely a contaminant from the upper respiratory tract or the cause of an exacerbation. During the thesis, isolates of *H. influenzae* were recovered from CF patients and examined by the classical phenotypic typing system of biotyping, and by genomic analysis (PFGE) to determine if certain strains are associated with pulmonary infection whilst others act as commensals.

Prior to commencing this study, it was necessary to develop a suitable method for extracting chromosomal DNA from isolates of *H. influenzae* and to select an appropriate restriction enzyme for DNA digestion prior to electrophoretic separation by PFGE.

Isolates of *H. influenzae* causing chronic lung disease and respiratory infection in non-CF adult patients are predominantly biotypes II and III (Brabender, 1984). Previous studies on biotyping of *H. influenzae* isolated from CF patients have produced conflicting results regarding the prevalence of these biotypes. Thus, it was decided to investigate the biotypes of isolates of *H. influenzae* from both non-CF and

CF patients to allow comparison between the distribution of biotypes within the two groups of patients.

3.3 *PSEUDOMONAS AERUGINOSA*

In the CF lung, *P. aeruginosa* generally supercedes *Staph. aureus* and *H. influenzae* and is responsible for exacerbations in the older child or adult patient. Early aggressive antipseudomonal treatment, when the organism is first cultured, may result in eradication. Unfortunately, this strategy only delays the onset of colonisation as the organism tends to return at a later date. The time interval between eradication and the re-emergence of *P. aeruginosa* varies depending on the patient. It may become increasingly difficult to eradicate the organism with each subsequent colonisation and a stage is reached when antimicrobial therapy fails to eradicate the organism although the load of *P. aeruginosa* may be reduced. Studies using phenotypic typing systems have shown that once a patient becomes chronically colonised with *P. aeruginosa* the same strain generally remains for the life of the patient. One of the aims of this section of the thesis was to perform a longitudinal study to compare the phenotypic and genomic fingerprints of the original isolate of *P. aeruginosa* with subsequent isolates from the same patient.

P. aeruginosa undergoes a striking transformation in the majority of CF patients who have been colonised for some time, with the classical non-mucoid colonial phenotype converting to a highly gelatinous mucoid variant. The time taken for this conversion differs from patient-to-patient and varies from as short as a few weeks to several

years. Although phenotypic fingerprinting, and CF 'tradition' has suggested that the mucoid organism is a clonal variant of the non-mucoid form, the transition has not been investigated to any great extent using more discriminating molecular typing techniques. Thus, PFGE was used to compare the DNA fingerprint of the original non-mucoid isolate of *P. aeruginosa* and the emerging mucoid phenotype.

In contrast to the notorious spread of *B. cepacia*, there is little evidence that *P. aeruginosa* is associated with cross-infection in CF patients, except in siblings and small clusters of patients within individual CF centres. Recently, however, a large number of CF patients attending a Liverpool clinic were found to be colonised with a ceftazidime resistant strain of *P. aeruginosa*. These isolates were investigated by bacteriocin typing and PFGE to determine if the outbreak is due to a common strain of *P. aeruginosa*.

3.4 *BURKHOLDERIA CEPACIA*

B. cepacia is a relatively recent pathogen to be isolated from respiratory secretions of CF patients, and only emerged as a problem in the UK in the late 1980s. However, in addition to innate multiresistance and virulence, *B. cepacia* is now a cause of great concern within the CF community, due to the ability of certain strains to be readily transmitted from patient-to-patient. For example, by the mid 1990s, the 'epidemic' or ET12 strain of *B. cepacia* was detected in 50% of CF patients in the UK (Pitt *et al*, 1996). Another *B. cepacia* issue is the association of certain strains with the life-threatening fulminant pneumonia known as 'cepacia syndrome'. Hence,

epidemiological studies form an essential part of surveillance to monitor the spread of *B. cepacia* among CF patients and to identify the individual strain of *B. cepacia* responsible for colonising an individual patient. An aim of this thesis was to investigate the efficacy of PFGE as a typing system for *B. cepacia*.

The first isolate of the 'epidemic' strain of *B. cepacia* from an Edinburgh CF patient was recovered in 1989. This patient remains colonised with *B. cepacia* ten years later. Using stored isolates from the Edinburgh strain repository, a longitudinal study was carried out on the isolates recovered over the period of colonisation to identify any change in the PFGE profiles produced during this time. Furthermore, a number of Edinburgh CF patients have acquired this 'epidemic' strain of *B. cepacia* over the last nine years and it is proposed to investigate isolates from each of these patients for any variation in the strain as it transfered from one patient to another.

In North America, there is an increasing interest in the agricultural use of *B. cepacia* for soil bioremediation and as a biological control agent against major fungal infections in commercial crops. Several studies have claimed that environmental and biopesticide isolates of *B. cepacia* differ from those causing infection in humans (Butler *et al*, 1995; Honicky *et al*, 1993). However, this issue is the subject of much debate. One of the aims of this section is to use PFGE to investigate the relationship between environmental and clinical *B. cepacia* and in particular to address the hypothesis that 'environmental' *B. cepacia* are responsible for colonisation of CF patients.

3.5 STENOTROPHOMONAS MALTOPHILIA

In recent years, *S. maltophilia* is recovered with increasing frequency from CF respiratory secretions. However, the reasons for this increase and the pathogenic significance of *S. maltophilia* remain unclear. The aims of this thesis were to investigate if cross-infection could explain the increased prevalence. For this purpose, isolates of *S. maltophilia* from paediatric and adult CF patients were examined by PFGE and their clonality determined. A second aim was to test the hypothesis that the increased incidence of this inherently resistant organism might arise from aggressive antipseudomonal treatment.

CHAPTER 4

MATERIAL AND METHODS

4.1 BACTERIAL STRAINS

Bacterial isolates used in this thesis were obtained from the Edinburgh CF Microbiology Laboratory culture collection, the Clinical Microbiology Department, Western General Hospital, Edinburgh or the Clinical Microbiology Department, Royal Infirmary of Edinburgh. Specimens of sputum and nasal swabs were obtained from CF patients attending the CF clinics at Royal Hospital for Sick Children and Western General Hospital, Edinburgh. Nasal swabs were also obtained from members of staff in Medical Microbiology Department, Edinburgh University Medical School.

4.2 CHEMICALS AND MEDIA

Unless otherwise stated, all chemicals were obtained from Sigma (Sigma-Aldrich Coy. Ltd., Poole, Dorset) and all media from Oxoid (Oxoid, Basingstoke, Hants).

Nutrient agar (NA): Columbia blood agar base 39 g/L.

Blood agar (BA): Columbia blood agar base, 39 g/L, with 5% defibrinated horse blood (Oxoid).

Chocolate agar (CBA): Columbia blood agar base, 39 g/L, with 5% defibrinated horse blood, heated in boiling water until the blood turns brown.

Chocolate agar plus bacitracin (CBA+): chocolate blood agar plus bacitracin (10 U/ml).

Tryptone soya agar (TSA): Tryptone soya agar 40 g/L.

Sensitivity test agar (STA): Diagnostic sensitivity test agar 40 g/L.

Pseudomonas isolation agar (PIA): Pseudomonas isolation agar (Difco Laboratories, Michigan, USA) 45 g/L with 2% (v/v) glyerol (BDH, Merck Ltd., Dorset) added prior to autoclaving.

Cepacia agar (CEP): Cepacia agar (Mast Diagnostics Ltd., Bootle) 32.5 g/L. Add one Selectatab (Mast) per 100 ml agar, after autoclaving and when agar has cooled to 50°C. This gives a final concentration of 300 U/ml polymyxin and 100 μg/ml ticarcillin.

Nutrient broth (NB): Oxoid nutrient broth No 2, 25 g/L.

Nutrient broth yeast extract (NBYE): Oxoid nutrient broth No 2, 25 g/L, plus 0.5% yeast extract (Difco) added prior to autoclaving.

Brain heart infusion broth (BHI): brain heart infusion powder (Difco) 37g/L.

Skim milk: Skim milk powder, 10% (w/v). Sterilised by autoclaving at 121°C for 5 min.

Saline: 0.85% sodium chloride in distilled water

Malka medium (Robert-Gero *et al*, 1970)

Stock solutions:

A Na_2HPO_4 (7.34 g/100ml), KH_2PO_4 (3.24 g/100ml). pH adjusted to 7.2.

Stored over 5 ml chloroform.

B $M_gSO_4.7H_2O$ (2.05 g/100ml). Stored over 5ml chloroform.

C 50% (w/v) glucose. Filter sterilised

D $FeSO_4.7H_2O$ (0.183 g/100ml). Add 1 drop of concentrated H_2SO_4 or HCl.

Sterilised by autoclaving for 15 min.

E $(NH_4)_2SO_4$ (5 g/100ml). Stored over 5 ml chloroform.

Add 20 ml A, 20 ml B, 5 ml C, 1ml D and 20 ml E to 934 ml sterile distilled water.

Indole medium (Cowan & Steel, 1965)

Kovac's indole reagent

p-dimethylamino-benzaldehyde	5g
isoamyl alcohol	75ml
HCl (concentrated)	25ml

Levinthal stock

Brain heart infusion broth (Difco) was made according to the manufacturers recommendations. BHI was boiled vigorously, 10% defibrinated horse blood (Oxoid) was added. The mixture was filtered through Whatman No 1 filter paper and sterilised by membrane filtration (Gelman Sciences, Northampton).

Equal volumes of the Levinthal stock and autoclaved peptone solution; 1% bacto-peptone (Difco) and 0.5% proteose peptone (Oxoid) were mixed and dispensed into sterile bijoux, prior to inoculation. The bijoux were incubated at 37°C overnight. The culture was mixed and 0.5 ml of Kovac's indole reagent was added. A pink reaction indicated a positive result.

Urea medium (Lautrop, 1960)

KH_2PO_4 0.1%

K_2HPO_4 0.1%

NaCl 0.5%

phenol red 1:100000

This solution was adjusted to pH 7.0 prior to autoclaving.

A stock solution of urea (20%) was sterilised by filtration and 1ml added to 20ml medium. The urea solution was dispensed into 1ml amounts in WR tubes prior to inoculation. The tubes were incubated at 37°C in a waterbath and results read after 2hr and overnight incubation. A read colouration was indicative of a positive result.

Ornithine decarboxylase test (Moller, 1955)

proteose peptone (Oxoid)	5g
beef extract (Gibco BRL)	5g
pyridoxal	5mg
glucose	0.5g
distilled water	1000ml
bromothymol blue, 0.2% soln.	5ml
cresol red, 0.2% soln.	2.5ml

The solution was adjusted to pH 6.0 prior to the addition of the indicators and then sterilised by autoclaving at 115^{o}C for 20min. A filter sterilised solution of L-ornithine hydrochloride was added to the medium to a final concentration of 1%. 1ml of the ornithine decaboxylase medium was dispensed into WR tubes and sterile liquid paraffin was added until a thick layer was formed on top of the medium. The medium was inoculated through the paraffin layer and incubated at 37^{o}C for up to five days, although examined daily. If decarboxylation occurs the medium turns a violet colour.

All media was steriled by autoclaving at 121^{o}C/15psi for 15 min, unless otherwise stated.

4.3 GENERAL BACTERIOLOGICAL METHODS

4.3.1 Storage, recovery and growth of bacteria

Bacterial colonies were suspended in skimmed milk and stored at -70^{o}C and were recovered by streaking out onto the appropriate media. *Staph. aureus* was inoculated onto BA, *H. influenzae* onto CBA, *P. aeruginosa* onto PIA or NA, *B. cepacia* onto CEP or NA and *S. maltophilia* onto NA. All plates were incubated at 37^{o}C overnight or for 48 h in the case of some isolates of *B. cepacia*. Isolates of *H. influenzae* were incubated under microaerophilic conditions (in the presence of 10% CO_2).

4.3.2 Recovery of *Staph. aureus* from nasal swabs and sputum specimens.

Nasal swabs

Swabs of the anterior nares, in transport medium, were inoculated by rotating the swab onto the surface of a BA plate to form a well. The inoculum was then streaked out and the plate incubated at 37^{o}C overnight. Colonies suspected as being *Staph. aureus* were confirmed by Staphaurex Plus reagent (Murex Biotech Ltd., Dartford, Kent) according to the manufacturers instructions.

Processing of sputum

An equal volume of Sputolysin (Calbiochem Novabiochem (UK) Ltd., Nottingham) was added to the sample of sputum and vortexed.

Sputum was then diluted in sterile saline from 10^{-1} to 10^{-4}.

100µl of each dilution: neat, 10^{-2} and 10^{-4} was pipetted onto the following culture media: BA, CBA+, PIA and CEP

The inoculum was spread across the surface of the plate using a sterile glass spreader.

The plates were then incubated at 37°C overnight aerobically, except CBA+ which was incubated in 10 % CO_2.

4.3.3 Making an isolate of *S. maltophilia* resistant to imipenem

Isolates C1931 and C1941 had a minimum inhibitory concentration (MIC) to imipenem of 2 µg/ml. STA plates containing imipenem at varying concentrations from 2 µg/ml to 40 µg/ml, in increments of 2 µg/ml, were made. Each isolate was inoculated into 10 ml NBYE and incubated overnight at 37°C in shaking incubator. The broth was diluted to 10^6 orgs/ml in saline, then 0.1 ml was spread across the surface of several STA plates containing different concentrations of imipenem. The same procedure was carried out with the isolates which grew at the highest concentration of imipenem until a final MIC was reached.

4.3.4 Test for DNase production in isolates of *S. maltophilia*

A suspension of each isolate was made in 1 ml saline and inoculated onto DNA medium (Mast Diagnostics, Bootle) using the multipoint inoculator (A400 Multipoint Inoculator, Denley, Billingshurst, Surrey). The plates were incubated at 37°C overnight. The plates were then flooded with N HCl and left for a few

minutes. DNase production is observed as an area of clearing, in the agar, around the inoculated site.

4.3.5 Maceration of onions by *B. cepacia*

The outer skin of the onion was removed and then the onion was cut into slices using a sterile scalpel. A medical wipe saturated in sterile distilled water was placed in a sterile petri dish, to create a moist environment, and a slice of onion placed on top. A few drops of an overnight NBYE culture of the test organism, grown at 37°C shaking, was pipetted onto the surface of the onion. An uninoculated onion slice in a petri dish was also incubated, as a negative control. The onion slices were incubated at 30°C for 7 - 10 days. The onions were examined on a regular basis for any signs of maceration.

4.3.6 Minimum inhibitory concentrations of antibiotics

Minimum inhibitory concentrations were determined by agar dilution method as described in the Journal of Antimicrobial Chemotherapy Volume 27, Suppl. D, 1991 page 28.

4.3.7 Inhibition of isolates of *H. influenzae* by *P. aeruginosa*

An isolate of *P. aeruginosa* was streaked down the centre of a CBA plate and incubated at 37°C overnight. The growth was removed using a slide dipped in chloroform. The plate was then exposed to chloroform for 15 min and exposed to the air for a further 15 min to allow any residual chloroform to evaporate. This

procedure was carried out in a Class I safety cabinet. The strains of *H. influenzae*, under investigation, were inoculated across the diameter of the plate, at right angles to the area in which the *P. aeruginosa* growth had been removed. The plate was again incubated at 37^{o}C overnight and then examined for any sign of inhibition of growth of the *H. influenzae* across the area in which the *P. aeruginosa* had grown.

4.4 PHENOTYPIC TYPING SYSTEMS

4.4.1 Bacteriocin typing

Bacteriocin or pyocin typing of *P. aeruginosa* was performed by the method of Fyfe *et al* (1984) using eight main and five sub-indicator strains.

Bacteriocin typing of *B. cepacia* was performed by the method of Govan & Harris (1986).

4.4.2 Biotyping

The work in this thesis used conventional biochemical testing methods for indole, urea and ornithine decarboxylase to biotype isolates of *H. influenzae*.

4.5 GENOMIC TYPING SYSTEMS

4.5.1 Pulsed-field gel electrophoresis (PFGE)

Buffers

SE buffer: 75mM NaCl, 25mM EDTA; pH 7.5.

Lysis buffer: 1% (w/v) N-lauroylsarcosine, 0.5M EDTA; pH 9.5.

TE buffer: 10mM Tris, 10mM EDTA; pH 7.5.

EC buffer: 6mM Tris-HCl, 1M NaCl, 0.1M EDTA, 0.5% Brij 58, 0.2% deoxycholate, 0.5% sarkosyl.

TEN buffer: 0.1M Tris-HCl, 0.15M NaCl, 0.1M EDTA.

Tris borate EDTA X5 concentrate (Sigma T-6400)

Preparation of chromosomal DNA

Method for the preparation of chromosomal DNA from *B. cepacia, P. aeruginosa,* and *S. maltophilia* was modified from that described by Vasil *et al* (1990). Cultures were grown in 10 ml NBYE overnight at 37°C with orbital incubation at 100 rpm. After centrifugation at 2500 x g for 10 min, bacteria were resuspended in SE buffer and standardised spectrophotometrically to an OD_{590} of 1.5. 0.5 ml of 1% low melt agarose (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts.), in SE buffer, was added to 0.5 ml of the suspension and pippeted into the plug mold. The mold was placed at 4°C for 15 min. to allow the plugs to solidify. The plug was placed in a bijoux containing 2 ml lysis buffer plus proteinase K, 0.5mg/ml (Sigma) and incubated at 55°C overnight. After the lysis step the buffer was replaced by washing three times in TE buffer and stored at 4°C until required.

Digestion of DNA involved cutting a portion of the plug and placing it in an Eppendorf tube together with 100 µl diluted restriction buffer; 2 µl bovine serum albumen (BSA), 10 mg/ml; 2 µl dithiothreitol (DTT), 1.5 mg/ml; 2 µl restriction enzyme *Xba*I (TCTAGA) or *Spe*I (ACTAGT) (Gibco BRL, Life Technologies Ltd.,

Paisley, Renfrewshire). After overnight incubation at 37°C, the plug was loaded into the well of a 1% pulsed-field agarose (Bio-Rad), prepared in 0.5 x TBE buffer. Electrophoresis was performed with the CHEF-DR II (Bio-Rad). The pulse times varied according to the organism involved but in each case electrophoresis was run for 20 h. The gel was then stained with ethidium bromide (0.5 μg/ml) (Bio-Rad) for 15 min. followed by 30 min. destaining in distilled water prior to examination by UV transillumination (UV Products, Cambridge). The photograph was taken using a Polaroid camera with a setting of F11 B for 2.5 min. using black and white film type 665 (Polaroid) and allowed to develop for 50 sec.

Further modifications were involved for each organism and those will be outlined in the relevant chapters.

Chromosomal DNA was prepared from *H. influenzae* by a modification of the method used by Vasil *et al* (1990). A suspension of the growth from an overnight culture of *H. influenzae* on CBA was made in 1 ml NB. After centrifugation (MSE Microcentaur) at 6,500 rpm for 2 min, the pellet was washed in 1 ml SE buffer and resuspended in 0.5 ml SE buffer. An equal volume of 1% low melt agarose, in SE buffer, was added and the suspension was pippeted into the plug mold. The plugs were placed in a bijoux containing 2 ml lysis buffer plus proteinase K, 0.5 mg/ml and incubated at 55°C overnight. After the lysis step the buffer was replaced by washing three times in TE buffer and stored at 4°C until required.

The method for the preparation of chromosomal DNA for *Staph. aureus* was modified slightly from that described by Bannerman *et al* (1995). Cultures were grown in 5 ml BHI broth (Oxoid) for 3hr and 0.7 ml of this culture was centrifuged (MSE Microcentaur) at 6,500 rpm for 2 min. Cells were washed once in 1 ml TEN buffer and resuspended in 0.3 ml EC buffer. 2 µl lysostaphin (1 mg/ml solution) dissolved in 20 mM sodium acetate was added to the cell suspension and vortexed. 0.3 ml of 2% low melt agarose, in EC buffer, was added to the cell suspension, mixed and pippeted into the plug mold. The plugs were allowed to solidify at room temperature for 15 min. and then placed into a bijoux, containing 3 ml EC buffer, and lysed at 37°C for 1h. The EC buffer was removed and 3 ml TE buffer added and incubated at 55°C for 1h. This buffer was then replaced with 3 ml TE buffer and stored at 4°C until required.

For electrophoresis, a portion of the plug was placed in an eppendorf tube to which 100 µl diluted restriction buffer, 2 µl BSA, 2 µl DTT and 2 µl *Sma*I (CCCGGG) was added. After 5 h incubation at 25°C the digested plug was loaded into the gel, as above. The running parameters were as follows: initial pulse time 5 s; final pulse time 40 s; voltage 200V for 20 h at 14°C.

4.5.2 PCR-ribotyping

Buffers

50 mM Tris-HCl (pH 8.0) **- 20 mM EDTA**

50 mM Tris-HCl (pH 8.0) **- 2 mM EDTA**

10 mM Tris-HCl (pH 7.5) **- 1 mM EDTA**

DNA preparation. Whole chromosomal DNA was purified by a modification of the method of Stull *et al* (1988). Cultures were grown overnight in 10 ml NBYE in orbital shaking incubator at 37°C. 0.7 ml of culture was centrifuged at 13,000 rpm for 2 min, washed in 1 ml 50 mM Tris-HCl - 20mM EDTA, and resuspended in 0.5 ml 50 mM Tris-HCl - 2 mM EDTA containing lysozyme (100 μg/ml) and incubated at 4°C for 15 min. Proteinase K and sodium dodecyl sulfate were added to a final concentration of 100 μg/ml and 0.5%, respectively. The mixture was incubated at 37°C for 2 h. The bacterial lysate was extracted with phenol:chloroform (1:1). Sodium acetate was added to a final concentration of 0.3 M, and two volumes of 95% ethanol were added. After a 2 h or overnight incubation at -70°C the precipitated DNA was recovered by centrifugation at 13, 000 rpm for 30 min and resuspended in 10 mM Tris - 1mM EDTA to give a final concentration of ~ 1 μg/ml.

PCR-ribotyping was performed by the procedure of Kostman *et al* (1992). The sequence of the primers used were: 16S, 5'-TTGTACACACCGCCCGTCA-3' and 23S, 5'-GGTACCTTAGATGTTTCAGTTC-3' (Crauchem, Glasgow). Amplification was performed in a mixture of 10 mM Tris-HCl (pH 8.8), 50 mM potassium

chloride, 1.5 mM magnesium chloride, 0.1% Triton X-100, 200 μM deoxynucleoside triphosphates, 100 pmol of each primer and 1 μl DNA template. 0.2 μl PrimeZyme DNA polymerase (Biometra Ltd., Maidstone, Kent) was added. The DNA was amplified during 30 cycles of PCR consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, except for the last cycle, during which the extension step lasted 4 min using Gene E thermocycler (Techne (Cambridge) Ltd., Duxford, Cambridge). Twelve microlitres of PCR product was analysed by electrophoresis at 150V for 1 h in a 1% molecular biology agarose (Bio-Rad). The gel was then stained by ethidium bromide and examined by UV transillumination.

4.6 STATISTICAL ANALYSIS

Statistical analysis was carried out using the chi-squared (χ^2) test with the following formula: the sum of $(O - E)^2 / E$, where O is the observed number and E the expected number. Having obtained a value for χ^2, and using the table of χ^2 distribution (Swinscow,1987) the corresponding probability is shown.

CHAPTER 5

STAPHYLOCOCCUS AUREUS

5.1 TAXONOMY AND NOMENCLATURE

Over a hundred years ago, cocci were first observed in diseased tissues and in pus obtained from human abscesses. These organisms were classified by Billroth (1874) on the basis of their cell arrangements as monococcos, diplococcos, streptococcos and gliacoccos. In 1880, a Scottish surgeon, Sir Alexander Ogston, showed conclusively that a cluster-forming coccus was the cause of certain pyogenic abscesses in man. At the same time, similar observations were made in France by Louis Pasteur. This pyogenic organism was named *Staphylococcus* by Ogston (1882); the name being derived from the Greek noun *staphlye* (a bunch of grapes) and *coccus* (a grain or berry). Ogston proposed this name for the cluster-forming cocci to distinguish them from the chain-forming streptococci classified by Billroth.

Rosenbach (1884) was probably the first to isolate and grow *Staphylococcus* in pure culture and to study its characteristics. This organism was isolated from pus and was considered identical to those observed and described by Ogston; Rosenbach adopted the name *Staphylococcus* for the genus. Two colonial types were observed, distinguished only by their colour: one being cream or yellow and named *Staphylococcus pyogenes aureus* (*Staph. aureus*) and the other white, named *Staphylococcus pyogenes albus* (*Staph. albus* or *Staph. epidermidis*). There are now

nearly thirty defined species of *Staphylococci* of which the three species, *Staph. aureus*, *Staph. epidermidis* and *Staph. saprophyticus* account for the majority of infections in man.

5.2 GENERAL CHARACTERISTICS

Staphylococci are non-motile, non-spore forming, catalase-positive Gram-positive spherical cocci which divide in more than one plane to form clumps. Their cell walls comprise two main components, peptidoglycan and teichoic acid, which are important cell-adherence factors. *Staph. aureus* contains an additional third component, protein A, an antiphagocytic factor, not present in other species of staphylococci, which is covalently incorporated into the cell wall. The peptidoglycan chains, within the cell walls, are linked by pentaglycine bridges which break under attack by lysostaphin causing lysis of the bacteria.

Staphylococci are facultative anaerobes but grow best under aerobic conditions requiring as many as 12 amino acids and growth factors for growth (Fildes *et al*, 1936). They are also able to grow under anaerobic conditions where they require uracil and a fermentable carbon source (Richardson 1936).

Staph. aureus is readily distinguished from *Staph. intermedius* by production of coagulase (Chapman, 1934, Cruickshank, 1937). The majority of strains possess "bound" coagulase on their outer surface which binds to fibrinogen causing organisms to aggregate in the presence of rabbit plasma. However, in some strains

only "free" coagulase is produced and this can only be detected by the clotting of plasma in a test tube. *Staph. aureus* ferment a wide range of carbohydrates; these include mannitol which can be incorporated into medium as a selective agent to assist in the isolation of *Staph. aureus* (mannitol salt agar).

Staph. aureus gives positive results to acetoin production (Voges-Proskauer), gelatinase, alkaline phosphatase and DNAse but does not produce indole. It can survive in the environment under moist and dry conditions, has the ability to grow in up to 15% sodium chloride, withstand heat at 60^{o}C for 30 min but is killed by phenolic and hypochlorite disinfectants.

Staph aureus produces a number of enzymes which may act as virulence factors; these include catalase, coagulase, nucleases, hyaluronidase, proteinase, phosphatase and fibrinolysin and several toxins including enterotoxins A-E, toxic shock syndrome toxin, epidermolytic toxins A & B and haemolysins alpha, beta, gamma and delta (Cohen, 1986). Almost all strains of *Staph aureus* produce toxic alpha haemolysin while beta haemolysin is more commonly associated with isolates cultured from animals.

Some strains of *Staph. aureus* have been shown to produce capsular polysaccharides (CPs) which have been classified into eight types (1 - 8) using monoclonal antibodies. The capsules cannot be detected by traditional methods such as India ink preparation or by the specific capsule reaction because of their small size (Fournier *et*

al, 1984). Encapsulated strains of *Staph. aureus* have been isolated from CF patients with a random distribution of capsular types. Antibody response to CP types 5 and 8, measured by ELISA, was not elevated in CF patients with chronic *Staph. aureus* lung infection; interestingly, the levels of antibody to CP 8 were significantly lower in comparison with healthy carriers (Albus *et al*, 1988)

5.3 PATHOGENESIS

Staph. aureus is the most important staphylococcal pathogen in humans. Human skin is densely colonised with coagulase negative staphylococci and to a lesser extent with *Staph aureus*; the latter is more commonly found in human anterior nares.

Staph aureus causes a wide variety of infections ranging from localised or superficial to systemic infections and toxin-mediated illnesses. Localised or superficial staphylococcal infections include skin infections, otitis-media, post-operative wound infections, osteomyelitis, bronchopneumonia, lung abscess, endocarditis and empyema. Systemic infections include septicaemia and "spontaneous" bacteraemia; these may occur without an obvious septic focus, particulary in patients debilitated by chronic hepatic or renal disease or by diabetes mellitus. Toxic shock syndrome and food-poisoning due to *Staph. aureus* are toxin-mediated illnesses.

For bacteria to colonise mucosal surfaces, they must first be able to attach to epithelial cells and once bound to evade immune clearance. Bacteria vary in their ability to attach to epithelial cells but *Staph aureus* has a high binding capacity for

such cells. Fibronectin, a glycoprotein found in biological fluids such as plasma, has been shown to bind to teichoic acid, a major structural component of the *Staph. aureus* cell wall, thus allowing the bacteria to adhere to epithelial cells (Aly & Levit, 1987). Teichoic acid together with staphylococcal slime, which is biochemically distinct from the alginate-like exopolysaccharide produced by *P. aeruginosa* (Campbell *et al*, 1986), play significant roles in the adherence of *Staph. aureus* to respiratory epithelial cells in patients with CF. Once bound, *Staph. aureus* produces a variety of virulence factors including protein A and leucocidins which lyse phagocytic cells.

Staph aureus together with *P. aeruginosa*, *Staph. epidermidis*, *Strep. pyogenes* and diphtheroids adhere to nasal mucosal cells where the staphylococci constitute the major components of the bacterial flora of the anterior nares. *P. aeruginosa* also demonstrates good attachment but is rarely found in the nares of healthy adults (Aly & Levit, 1987). Why the anterior nares are colonised by *Staph. aureus* in some individuals or carriers is not known; however, microorganisms from carriers appear to adhere readily to nasal epithelial cells *in vitro*. Another possible reason for nasal colonisation by *Staph. aureus* may be minor deformities of the nasal cavity (Jacobs *et al*, 1961).

There appears to be three groups of individuals with respect to nasal carriage of *Staph. aureus*: persistent carriers, persistent non-carriers and transient carriers (Hu *et al*, 1995). Bacterial fingerprinting by PFGE demonstrated that carriers may harbour

the same strain of *Staph. aureus* in their nose for over a year while others may be colonised intermittently. Carriage appears to vary according to age with the majority of babies carrying *Staph. aureus* in their noses, within a few days of birth (Aly & Levit, 1987). Carriage decreases with increasing age and usually reaches adult levels by the age of five to six years (Williams, 1963). A study in the Netherlands showed that carriage varied from 40% in individuals under 20 years, to 30% in those over 20 years, 0% in 60 year olds and 15% in those over 70's (Noble, 1967). A higher carriage rate (approximately 58%) was observed in hospitalised patients or individuals working in hospitals (Miles *et al*, 1944).

Methicillin-resistant *Staph. aureus* (MRSA) is one of the most frequent agents of nosocomial infection. Although these organisms have not been shown to be more virulent than other *Staph. aureus*, their multiresistance makes them responsible for higher mortality rates in compromised patients (Locksley *et al*, 1982).

5.4 *STAPH. AUREUS* IN CF PATIENTS

Staph aureus is generally the initial bacterial pathogen detected in respiratory secretions from CF patients, especially young children, and is usually followed by *P. aeruginosa*. The precise reasons for this progression are not known although an attempted explanation is the adhesion of both pathogens to asialoganglioside GM1 (aGM1) epithelial receptors and the increased population of aGM1 in regenerating epithelium (Prince, 1992; De Bentzmann *et al*, 1996). Before the antibiotic era, lung infection due to *Staph. aureus* was the major cause of mortality in CF patients

(Anderson, 1938). For example, in 1946, di Sant 'Agnese reported *Staph. aureus* as the main organism cultured from the respiratory tract in 11 out of 14 cases on post-mortem examination. Although antibiotic therapy is probably a major factor contributing to the decline of staphylococcal infections, particularly in adult CF patients, bacterial antagonism by *P. aeruginosa* may play an important role. Machan *et al* (1991) have shown that some strains of *P. aeruginosa* produced two substances which inhibit the growth of *Staph. aureus*. One of the substances was pyocyanin, a pigment produced by *P. aeruginosa*, and known to be an antibacterial agent (Schoental, 1941) while the other was present in non-pigmented strains of *P. aeruginosa*, was soluble in chloroform, heat stable and showed strong anti-staphylococcal activity which included MRSA.

Staph. aureus has the ability to adhere to respiratory epithelial cells and once bound to evade immune clearance. Together with *P. aeruginosa*, *Staph. aureus* also has an affinity for CF mucin and this property together with abnormalities of mucociliary clearance in the CF lung may contribute to persistent infection and progressive pulmonary damage. The two pathogens do not, however, share the same mucin receptors (Ramphal, 1990).

Interestingly, *Staph. aureus* has been found to adhere better to a CF bronchial cell line (genotype ΔF 508/w1282X) than to control cells (Imundo *et al*, 1995). CF patients overproduce a particular ganglioside (aGM1) on the surface of epithelial

cells and increased binding of *Staph. aureus* to the tetrasaccharide (Galβ1-3GalNacβ1-4Galβ1-4Glc) of aGM1 has been found (Imundo *et al*, 1995).

Early aggressive anti-staphylococcal therapy is essential to prevent lung damage in CF patients (Marks, 1990). Many CF centres use long term prophylatic antistaphylococcal therapy from the first diagnosis of CF but this strategy remains controversial and may lead to two major problems. First, continuous anti-staphylococcal treatment results in a more rapid emergence of *P. aeruginosa* colonisation or infection (Bauernfeind *et al*, 1987, Geddes, 1988) and the emergence of mucoid variants (Kulczychi *et al*, 1978). Second, the emergence of *Staph. aureus* resistance, in patients receiving long-term cotrimoxazole arises due to the uptake of thymidine from the environment resulting in thymine-dependant strains. These organisms produce typical colonial morphology on mannitol salt agar but fail to grow on normal isolation media. In a study by Gilligan *et al* (1987), 21% of the CF patients were infected with thymidine-dependant *Staph. aureus*. These organisms could be easily missed if the use of mannitol salt agar was not used routinely. The strategy of prophylactic anti-staphylococcal therapy remains controversial. The use of long-term anti-staphylococcal prophylatic treatment showed significantly higher hospital admission rates in CF patients not receiving flucloxacillin than those treated with flucloxacillin prophylatically (Weaver *et al*, 1994). However, in Edinburgh, the policy of only treating patients presenting clinical evidence of exacerbations and *Staph. aureus* greater than 10^6 cfu/ml in their sputum may explain the relatively low incidence of *P. aeruginosa* (Govan *et al*, 1987).

Methicillin resistant *Staph. aureus* are recovered infrequently from respiratory secretions of CF patients (Bauernfeind *et al*, 1990). A study by Boxerbaun *et al* (1988) showed no recovery of MRSA in the years 1984 and 1985 but 14 cases in 212 CF patients occured in 1986. Recovery of MRSA from individual patients was not associated with any clinically significant outcomes and the patients did not receive any specific antimicrobial treatment. Of the 14 MRSA positive patients, 10 spontaneously lost the organism while four remained colonised without any adverse results.

5.5 EPIDEMIOLOGY AND TYPING SYSTEMS

Staph. aureus is a major cause of nosocomial and community-acquired infections and the second most persistent pathogen isolated from respiratory secretions of CF patients. In the management of non-CF patients, it is important to determine the relatedness of staphylococcal isolates when investigating an outbreak and to consider the possibility of carriers acting as a source of nosocomial infection. In CF patients, epidemiological studies of *Staph. aureus* could determine whether the same strain or different strains are responsible for recurrent bronchopulmonary infections. Numerous typing systems are available for epidemiological studies of *Staph. aureus*. Phenotypic typing systems, including phage typing, are easy to perform and interpret while systems involving DNA probing, include ribotyping, restriction fragment length polymorphism (RFLP) and insertion sequence (IS) typing are labour intensive and time consuming. However, phenotypic markers are often unstable and evidence suggests that genomic typing systems are more reliable.

5.5.1 Bacteriophage typing

Traditionally, bacteriophage typing (Blair & Williams, 1961) has been the most widely used fingerprinting system for epidemiological studies of *Staph. aureus*, although its use has been mainly restricted to reference laboratories. Phage typing has been used in the CDC (Communicable Disease Center, Atlanta) for over 30 years and has several known weaknesses; these include poor reproducibility and the problem that around 20% - 30% of strains are untypeable (Tenover *et al*, 1994; Schlichting *et al*, 1993; Bannerman *et al*, 1995). Maintenance of the large number of phage stocks and propagation strains is also laborious. However, phage typing shows a high degree of discrimination; it has been observed that isolates with identical genotypes, using PFGE, may produce different phage types (Branger *et al*, 1996). Similar findings were observed using field inversion gel electrophoresis analysis of RFLP where isolates with similar but not identical profiles, suggesting relatedness of the strains, were shown to have different phage types (Goering *et al*, 1990).

In contrast, PFGE identified subgroups within a phage group and is thus more discriminating than bacteriophage typing (Bannerman *et al*, 1995). This result combined with the other weaknesses of phage typing has led the CDC to convert from phage typing to the use of PFGE for typing strains of *Staph. aureus*.

5.5.2 Biotyping

Biotyping using the method of Hebert *et al* (1988) works well for coagulase-negative staphylococci but produces too many subtypes when used for *Staph. aureus* and does not produce good correlation with epidemiological data or other typing methods.

5.5.3 Capsular serotyping

Capsular serotyping (Arbeit *et al*, 1984) divides *Staph. aureus* into eight types 1 - 8 but has a very low power of discrimination and has a similar number of untypeable strains to phage typing. However, good correlation was found between this method and esterase electrophoretic typing in a study of *Staph. aureus* in CF patients (Branger *et al*, 1994).

5.5.4 Esterase electrophoretic typing

Esterase electrophoretic typing (EET) or zymotyping (Branger & Goullet, 1987) involves electrophoretic mobility patterns of esterases produced in lysostaphin-treated staphylococcal cultures. Zymotyping is stable and reproducible. Epidemiological studies of *Staph. aureus* in CF patients showed that no specific zymotype was associated with CF and the same zymotypes were found in both CF and non-CF patients, however, slightly more diversity occured among the CF patients (Branger *et al*, 1994). EET is less discriminating than PFGE as isolates of *Staph. aureus*, previously characterised by EET, with identical PFGE profiles did not differ in EET but isolates with identical esterase typing belonged to different genotypes (Schlichting *et al*, 1993).

5.5.5 Plasmid analysis

Plasmid restriction analysis (Archer & Mayhall, 1983) was the first DNA-based method to be applied to *Staph. aureus* and is probably the easiest and most inexpensive of the genomic systems. Although used extensively, it is dependant on the presence and stability of staphylococcal plasmids. Specificity of the technique is high but the reproducibility poor (Tenover *at al*, 1994). CF patients found to be infected with *Staph. aureus* on only one occasion revealed no predominant plasmid or lysotype whereas five patients, who were colonised for over 10 months, had identical strains when examined by phage and plasmid profile typing techniques (Goering *et al*, 1990).

5.5.6 Ribotyping

Ribotyping is less discriminating than PFGE for typing strains of *Staph. aureus* (Prevost *et al*, 1992). Ribosome spacer DNA amplicon polymorphism or RS-PCR is a more rapid method of ribotyping, is relatively inexpensive, highly reproducible and almost as discriminating as PFGE (Kumari *et al*, 1997).

5.5.7 Insertion sequence typing

Insertion sequence typing (Barberis-Maino *et al*, 1987) was primarily designed to type MRSA and is relatively effective. However, this system is fairly ineffective for typing methicillin sensitive strains of *Staph. aureus*. Additional discrimination may be achieved using multiple probes but this adds extra expense and makes the system more labour intensive.

5.5.8 Immunoblotting

Immunoblotting (Tsang *et al*, 1983) is easy to perform and was successful at distinguishing outbreaks from unrelated strains of *Staph. aureus,* as long as the subtypes were ignored. Using a stricter interpretation of results produced a much lower degree of sensitivity. Interpretation of the complex banding patterns are difficult for inexperienced individuals. One of the disadvantages of this system is the use of human serum for Western blots.

5.5.9 PFGE

Isolates of *Staph. aureus* from two epidemiologically unrelated groups (one in France and the other in Germany) were genotyped by PFGE. Results showed that three strains of *Staph. aureus* with identical genotypes were present in both groups of patients (Schlichting *et al*, 1993). As there was no contact between the two groups, these findings demonstrate that the appearance of identical genotypes does not necessarily indicate an epidemic situation. Similar findings were observed by Branger *et al* (1996) in that isolates of *Staph. aureus* with identical genotypes were found in CF patients attending the same or different hospitals but there was no evidence of contact among those patients.

PFGE was used to provide evidence of possible transmission of a strain of *Staph. aureus* from a CF patient to four other CF patients, attending a four-week summer camp (Schlichting *et al*, 1993).

PFGE is the most discriminating method of typing isolates of *Staph. aureus* but there remains the problem of determining the number of band differences allowed before judging isolates as clonally unrelated. Branger *et al* (1996) has shown that consecutive isolates of persistent *Staph. aureus*, causing chronic or recurrent infections in CF patients, may demonstrate variant PFGE patterns differing by more than three bands due to several genetic random events, whereas other strains showed identical patterns.

5.6 COMPARISON OF TYPING SYSTEMS

Isolates of *Staph. aureus* from epidemiologically unrelated sources were investigated using the genomic typing system of PFGE compared with established and commonly used phenotypic methods such as zymotyping, capsular serotyping and bacteriophage typing (Schlichting *et al*, 1993). Comparison of the typing techniques revealed that capsular typing showed the poorest discrimination followed by zymotyping or esterase electrophoretic typing and phage typing with PFGE being the most discriminating typing system.

Tenover *et al* (1994) described an epidemiological study of 59 isolates of *Staph. aureus* and one strain of *Staph. intermedius* which determined the strengths and weaknesses of 12 available typing systems ranging from simple phenotypic systems to more technically demanding genomic methods. Each system was assessed according to the five criteria set out by Maslow *et al* (1993): typeability, reproducibility, disciminatory power, ease of interpretation and ease of use. The

methods involved were antibiograms, biotyping, bacteriophage typing, immunoblotting, insertion sequence typing (IS), multilocus enzme electrophoresis, restriction analysis of plasmid DNA, PFGE, field inversion gel elctrophoresis, restriction analysis of PCR-amplified coagulase gene sequences, RFLP using four staphylococcal genes as probes and ribotyping. Tenover *et al* (1994) found that no single method was superior to the others but biotyping produced numerous subtypes and was not effective at grouping outbreak-related strains of *Staph. aureus*. It was suggested that a combination of two methods may be most efficacious: one method to screen isolates and the other for detailed strain differentiation.

5.7 AIMS

Staph aureus is a commensal of the anterior nares in many healthy individuals, appearing at higher levels in infants and young children than in adults (Gould & McKillop, 1954). Several studies have investigated the incidence of carrier status in different groups of people but CF patients do not appear to have been included. Thus it was proposed to obtain nasal swabs from paediatric and adult CF patients and determine the carriage rate of *Staph. aureus* within this group of individuals.

During a Phase 1 gene therapy trial undertaken at the Western General Hospital, Edinburgh, eight patients received local application of the liposome/gene complex to nasal epithelial cells and eight patients received a placebo. Specimens of sputum were taken prior to treatment, on the day of treatment and at intervals of 3, 7, 21 and 28 days after treatment. Swabs of the anterior nares were taken 7 days before and 7

days after treatment with the exception of three patients who also had swabs taken on the day of treatment. The aim was to examine nose swabs to determine whether the delivery of the gene/liposome complex to the nose had any affect on the nasal flora, with particular interest in *Staph. aureus*. Further nasal swabs, from this group of patients, were examined to determine whether the anterior nares remained colonised with the same strain of *Staph. aureus* for any length of time.

As *Staph. aureus* is commonly found in the nose and is a common pathogen in respiratory secretions of CF patients, the rather obvious hypothesis was addressed that *Staph. aureus* from the nose was responsible for colonising the lower respiratory tract and causing pulmonary infection in CF patients. To answer this hypothesis, isolates of *Staph. aureus* from the anterior nares and sputum from the same patient were genotyped by PFGE to determine the relatedness, if any, between the isolates from the two sources.

5.8 RESULTS

5.8.1 Nasal carriage

One hundred and twenty one swabs of the anterior nares were sampled from 87 CF patients; 79 swabs from 49 (56%) paediatric patients and 42 swabs from 38 (48%) adult patients. Identification of isolates of *Staph. aureus* recovered from nasal swab cultures were confirmed by Staphaurex Plus. *Staph. aureus* was recovered from 16 of the 49 paediatrics and from 26 of the 38 adult CF patients, indicating a carriage rate of 33% in paediatric and 68% in adult patients, respectively.

A similar study was undertaken among members of staff in the Medical Microbiology Department at Edinburgh University, to determine the carriage rate in healthy individuals. Forty nasal swabs were collected and examined for the presence of *Staph. aureus*. The incidence of nasal carriage of *Staph aureus* in this group of individuals was surprisingly low at 25 %.

5.8.2 Fingerprinting isolates of *Staph aureus* by PFGE.

Isolates of *Staph. aureus* were typed by PFGE using the method described by Bannerman *et al* (1995). However, the density of the DNA fragments together with smearing within the lanes indicated that the concentration of DNA was too high. This problem was rectified by reducing the incubation period of the broth culture from overnight to 3h. The DNA digestion time using restriction enzyme *Sma*I (CCCGGG) was increased from 2h to 5h at 25°C, as this produced clearer banding patterns. Once the optimum conditions for PFGE separation of chromosomal DNA for *Staph. aureus* were achieved, numerous isolates from CF and non-CF patients were examined to assess the discriminatory powers of this technique. Figure 5.1. shows a range of PFGE profiles produced by *Staph. aureus* isolated from nine CF patients. *Sma*I digestion of DNA from *Staph. aureus* produced 15-18 fragments with a size range of < 48.5kb to 630.5kb.

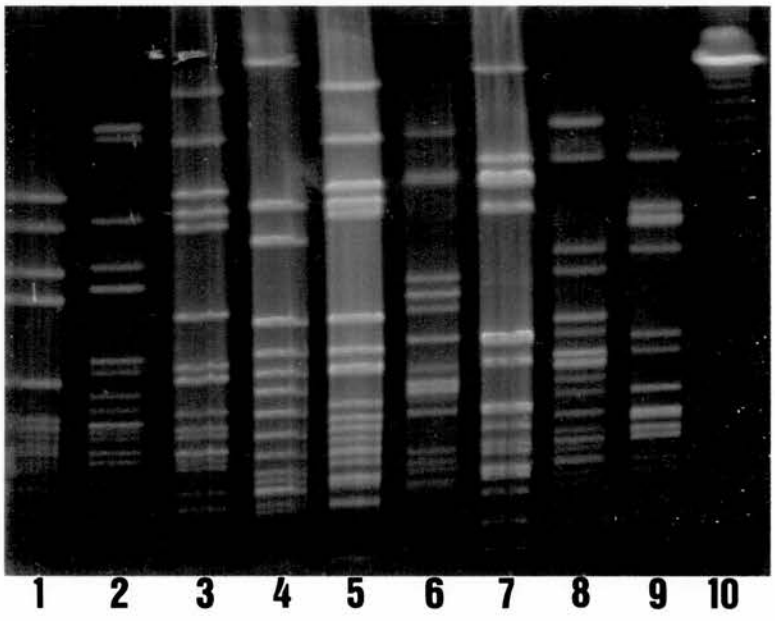


FIG. 5.1. *Sma*I digestion of DNA from nine isolates of *Staph. aureus* (lanes 1 to 9) showing heterogeneity of PFGE profiles. Lane 10, lambda concatemers (size range 48.5 kb to 630.5 kb, in increments of 48.5 kb).

5.8.3 Affect of local application of the liposome/gene complex on nasal flora.

Of the 16 CF patients involved in the gene therapy trial, *Staph. aureus* was isolated from nasal swabs in 15 patients on at least one occasion. Comparison of isolates of *Staph aureus* taken before and after the nasal application of gene therapy was possible in only nine of the 15 CF patients. Of the remaining six patients, only a single isolate of *Staph. aureus* was obtained from three patients and no *Staph. aureus* was present in the pre-treatment swabs from the other three patients. Subsequent, PFGE fingerprinting of the 18 nasal isolates of *Staph aureus*, from the nine CF patients, revealed the same strain of *Staph. aureus* was present before and after treatment in four of the nine patients while the remaining five patients harboured different strains of *Staph. aureus*.

5.8.4 Is the same strain of *Staph. aureus* present in both the nose and sputum?

As specimens of sputum were taken at the same time as the nasal swabs during the gene therapy trial, it was decided to compare the isolates of *Staph. aureus* from both sources. *Staph. aureus* was cultured from sputum only in seven of the 16 CF patients. Comparison of the PFGE profiles of the sputum isolates with those from the corresponding nasal isolates proved interesting. In four of the seven patients, isolates of *Staph. aureus* from the nose and sputum were found to be different. Patients 5 and 6 had two different strains of *Staph. aureus* present in the nose and in each case one of the nasal isolates was identical to the isolate present in the sputum. Patient 7 produced a particularly interesting result (Fig. 5.2). In this patient, *Staph. aureus* was detected from the anterior nares at day 7 (pre-treatment) and day 7 (post

treatment) and both isolates produced identical PFGE profiles, indicating that they were the same strain. This strain was not isolated in subsequent nasal swabs. Surprisingly, the original sputum isolate of *Staph. aureus*, taken prior to gene therapy treatment, appeared to have been replaced by the nasal isolate on treatment day and was still present three days later. However, the original sputum isolate returned at day 7 (post treatment) and was still present 28 days after treatment together with an additional strain. In fact, the original strain of *Staph. aureus* was still present in the sputum two years later. Meanwhile, the strain common to the nose and sputum was not detected in any of the specimens taken seven days post-treatment or later.

These results suggest the unexpected fact that the majority of the isolates of *Staph. aureus* found in the nose of the CF patient are different to those present in the sputum. Could nasal application of the liposome/gene complex be responsible for this phenomenon? To answer this question it was necessary to obtain isolates of *Staph. aureus* from other CF patients, not involved in the gene therapy program. Isolates of *Staph. aureus* were obtained from nasal swabs and sputum from 17 CF patients. Both specimens were taken on the same day, where possible, but otherwise within a few days of each other. Once again, PFGE revealed that different strains of *Staph. aureus* were present in the nose and sputum of individual patients, in this case in 13 of the 17 CF patients. Figure 5.3. shows four such CF patients, demonstrating the difference between isolates of *Staph. aureus* recovered from the nose and sputum.

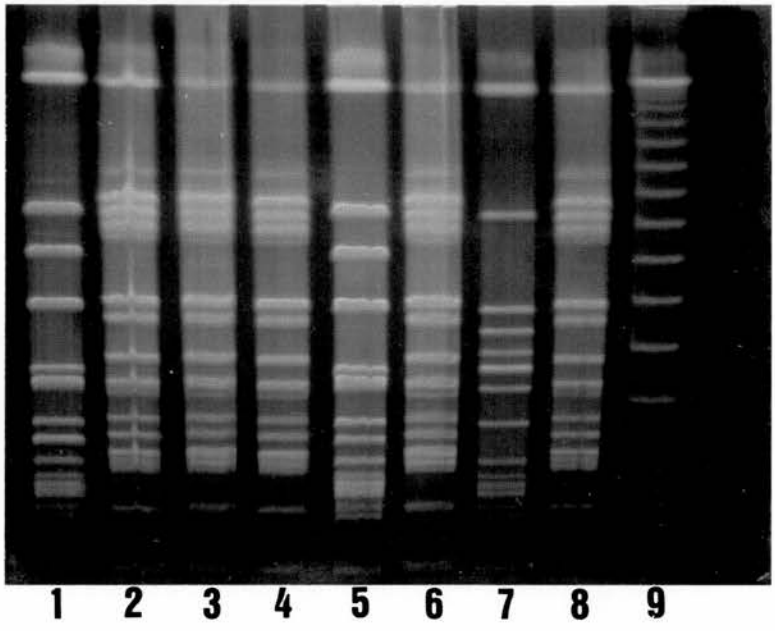


FIG. 5.2. *Sma*I digestion of DNA from *Staph. aureus* isolated from sputum and nasal swabs in a CF patient undergoing gene therapy. Lanes: 1, 3, 4, 5, 7, are sputum isolates taken at -14 days, TD, +3 days, +7 days, and +21 days, respectively. Lanes: 2, 6, and 8 are nasal isolates taken at -7 days, +7 days, and a year later, respectively. Lane 9. lambda concatemers (size range 48.5kb to 630.5kb, in increments of 48.5kb).

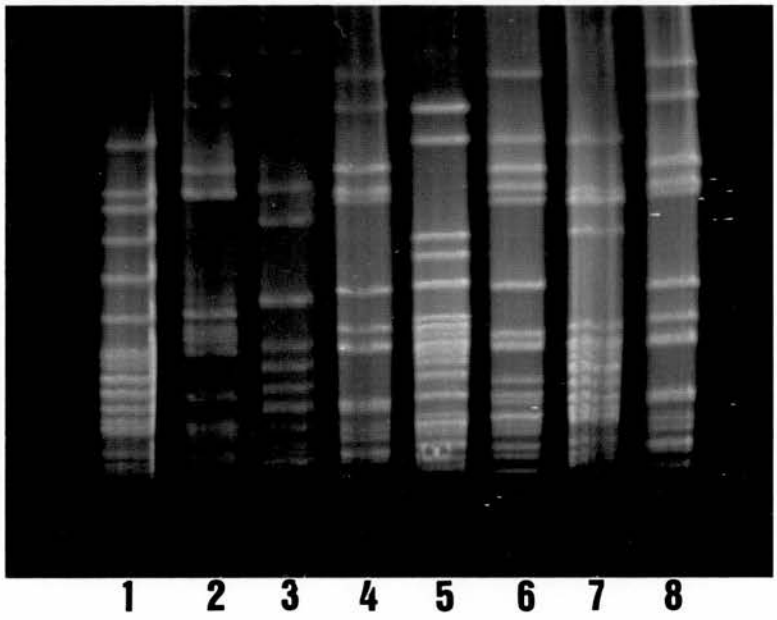


FIG. 5.3. *Sma*I digestion of DNA from *Staph. aureus* isolated from the nose and sputum in four different CF patients. Lanes: 1 to 8, P-A n/s, sputum; P-B n/s, sputum; P-C n/s, sputum; P-D n/s, sputum, respectively.

5.8.5 Does the same strain of *Staph. aureus* remain in the nose for any length of time?

It was decided to investigate the 15 CF patients involved in the gene therapy trial to determine whether the same strain of *Staph. aureus* remains in the nose for any length of time. Unfortunately, six of the CF patients were recruited from Glasgow and no further specimens were available. *Staph. aureus* was recovered from nasal swabs in only five of the nine remaining CF patients. Based on PFGE analysis, the results obtained from the 5 patients showed that the same strain of *Staph. aureus* was present in the nose of 2 patients more than a year later and eight months later in a third patient. The remaining two patients had two different strains of *Staph. aureus* during the gene therapy trial and in both cases the isolates recovered a year later were different to any previous isolate.

5.9 DISCUSSION

The primary reservoir of *Staph. aureus* is thought to be the anterior nares, which is the most consistent area from which this organism can be isolated (Williams, 1963). A recent review correlated the results of many studies on the incidence of nasal carriage undertaken over several decades and calculated that the mean rate of carriage of *Staph. aureus* in healthy individuals is 37.2 % (Kluytmans *et al*, 1997). Surprisingly at 25%, the rate of nasal carriage in members of staff in the Medical Microbiology Department at Edinburgh University is relatively low considering a significant proportion of individuals are exposed to *Staph. aureus* in their working environment. However, comparison of carriage rates between healthy individuals

and CF patients showed a marked difference; with a significantly ($p<0.001$, χ^2 test) higher carriage in adult CF patients (68%) compared to 33% in CF patients under 16-years old. Interestingly, carriage in the latter group is virtually the same as in the healthy non-CF population.

A possible explanation for the high nasal carriage rate in CF adults, could be the increased affinity of *Staph. aureus* for adherence to CF epithelial cells. However, this hypothesis does not explain the difference between paediatric and adult CF patients.

The diversity in PFGE profiles produced by *Staph. aureus* is less than that produced by other pathogens associated with pulmonary infection in CF patients. This general lack of heterogeneity became apparent when examining PFGE profiles of isolates of *Staph. aureus* from different patients. The same 'strain' was found among CF patients, who had no possible contact with one another, and also common strains were found among CF and non-CF patients. Similar findings were observed by Schlichting *et al* (1993), who found common strains of *Staph. aureus* in two groups of unrelated patients in France and Germany.

The nose has long been regarded as the major site of *Staph. aureus* carriage and the source from where the organisms can spread to other parts of the body (White, 1963). This dogma has been supported by epidemiological studies (Kluytmans *et al*, 1997; Nguyen *et al*, 1999). It was therefore assumed that the nose of a CF patient would

act as a reservoir for *Staph. aureus* with the potential to spread to the respiratory tract causing pulmonary infection. However, since PFGE data indicated that 76% of the patients in this study had different strains in the nose and sputum it would appear that pulmonary infection in the majority of CF patients is caused by *Staph. aureus* acquired from another source. Studies on an individual CF patient revealed that a nasal isolate of *Staph. aureus* appeared in the sputum and remained for a few days but was then eradicated and the original isolate returned. Analysis of subsequent specimens showed that the same strain of *Staph. aureus* can remain in the respiratory tract for at least two years. These results suggest that infection with a particular strain of *Staph. aureus* prevents further acquisition of a different strain.

The application of the liposome/gene complex to the nasal epithelial cells appeared to have little affect on the nasal flora in the CF patients. In some patients, the isolate of *Staph. aureus* in the nose before and after treatment was the same while in others the isolates were different. As none of the isolates of *Staph. aureus* in different patients were the same strain, it would appear that the invasive treatment associated with gene therapy was not responsible for the isolation of different strains of *Staph. aureus* in the nose.

CHAPTER 6

HAEMOPHILUS INFLUENZAE

6.1 TAXONOMY AND NOMENCLATURE

The genus *Haemophilus* is based on the 'influenzae bacillus' discovered by Pfeiffer in 1892. During a major outbreak of influenza, which took place between 1889-1892, Pfeiffer examined sputa from affected patients and found a similar type of bacillus in each case; the organism was absent in individuals who had not acquired influenza. He concluded that the bacillus was the causative agent for influenza and named it *Haemophilus influenzae*. In 1917, the organism was officially named *H. influenzae* by the classification committee of the Society of American Bacteriologists (Winslow *et al*, 1917). Subsequently, there was intermittent controversy as to whether Pfeiffer's bacillus was the cause of influenza or a secondary invader; this doubt was resolved in 1933 when Smith *et al* established that influenza was caused by a virus. After this discovery, interest in the genus *Haemophilus* declined but was revived in the 1970's when *H. influenzae* was confirmed as a pathogen causing a range of infections in man and various animal species (Kilian, 1976).

6.2 GENERAL CHARACTERISTICS

H. influenzae is a small non-motile, non-sporing, Gram-negative bacillus and shows marked pleomorphism varying from a cocco-bacillus to a long filamentous form. Some strains are facultative anaerobes in that they require 5-10% carbon dioxide for

growth but the majority of strains grow aerobically. *H. influenzae* ferments glucose, deoxyribose and xylose, with gas production in some strains, but not sucrose, lactose or mannitol. They reduce nitrates to nitrite and are catalase positive and oxidase positive. Identification of *Haemophilus* species is dependant on the requirement for the growth factors X and V, with some species requiring only a single factor while others, notably *H. influenzae* require both factors. X factor is heat-stable and is found in haemin or other iron- containing porphyrins. X dependant strains are unable to convert aminoalaevulinic acid to protoporphyrin. On the other hand, V factor is destroyed by heating to 120^{o}C but is released from red blood cells by gentle heating or can be replaced by coenzyme I and II or by nicotinamide dinucleotide.
H. influenzae demonstrate the phenomenon of 'satellitism' when grown in the presence of *Staph. aureus* on blood agar. Satellitism is recognised when colonies of *H. influenzae* grow larger around *Staph. aureus* as the latter secrete V factor into the medium to join X factor already present in the blood.

Immunoperoxidase staining for *H. influenzae* with monoclonal antibodies directed against outer membrane proteins provides a higher detection rate than cultural techniques (Moller *et al*, 1992); however, this procedure is not used routinely.
In general, infections due to *H. influenzae* are effectively treated with antibiotics, however, the use of β-lactam antibiotics can result in spheroplastic forms of *H. influenzae* which do not grow on standard culture media (Roberts *et al*, 1984). These spheroplastic forms revert to normal forms of *H. influenzae* if cultured on media supplemented with N-acetyl-D-glucosamine (Roberts *et al*, 1984).

6.3 PATHOGENICITY

In pathogenic terms, *H. influenzae* is the most important species of the genus *Haemophilus* and only affects man. *H. influenzae* and other *Haemophilus* species form part of the normal commensal flora of the mucous membranes of the throat, oral cavity and to a lesser extent in the genital tract in normal healthy individuals. However, following a viral infection, the organism can act as an opportunist pathogen causing secondary infections in the respiratory tract.

In 1931, Pittman identified the presence of capsulate and non-capsulate forms of *H. influenzae* and subdivided the former group into six capsular types, a - f, distinguishable by serotyping based on the antigenic nature of the capsular polysaccharide.

Capsulate strains, especially type b, are the most virulent form of *H. influenzae* and are responsible for invasive infections, and account for 95% of cases of meningitis, epiglottitis and bacteraemia associated with this organism. The presence of the capsule is a major virulence factor in *H. influenzae* serotype b although other pathogenic factors may exist (Moxon, 1992).

Non-capsulate strains of *H. influenzae* colonise the nasopharyngeal region in up to 80% of healthy people (Turk, 1984) but they can also play a pathogenic role. Non-capsulated *H. influenzae* is the cause of acute, recurrent and persistant infections of the mucous membranes in humans including lower respiratory tract infections in

patients with chronic obstructive pulmonary disease (Groeneveld, 1988). Non-capsulate *H. influenzae* is also an important pathogen in both children and adults with CF (Murphy *et al*, 1987) and a cause of otitis media, sinusitis, pneumonia and various infections in immunoglobulin-deficient patients. Musher *et al* (1983) reported seriological evidence to suggest that non-capsulate strains of *H. influenzae* may be the causative agents of pneumonia and chronic bronchitis by demonstrating a host antibody response to the patient's infecting organism.

Non-capsulate *H. influenzae*, responsible for respiratory disease, was thought to be merely a capsulate strain that had lost its capsule during bronchial invasion; however, capsulate strains appear to play no role in bronchiectasis. Another explanation for non-capsulate phenotypes was that the capsule was lost during passage *in vitro*. Gyorkey *et al* (1984), however, showed that non-capsulate strains are present *in vitro* and *in vivo*. In the early 1980's, several reports established clear distinctions between non-capsulate and capsulate type b strains of *H. influenzae*. These organisms affect different patient populations, cause different infections, present different surface antigens to the host and are genetically different. However, PCR has been used to show and confirm that a non-serotypable strain of *H. influenzae* may be derived from a type b capsulate strain (Jordens *et al*, 1993).

Fimbriae are present on the surface of many Gram-negative bacteria including *H. influenzae*. Their presence is not always associated with virulence but there is clear evidence that fimbriae function as adherence factors in many bacteria, thereby

enhancing bacterial colonisation of mucosal surfaces (Beachey, 1981). The role of fimbriae in the pathogenesis of infection caused by *H. influenzae* is unknown. They are, however, found in both capsulate and non-capsulate strains of *H. influenzae* recovered from the respiratory tract of adults (Apicella *et al*, 1984).

6.4 *H. INFLUENZAE* IN CF PATIENTS

Staph. aureus is generally held to be responsible for most of the initial chronic lung infections in young CF patients and is subsequently followed by *H. influenzae* and *P. aeruginosa*. It has been suggested that prophylatic treatment against *Staph. aureus* increases the incidence of *H. influenzae* and *P. aeruginosa* but this hypothesis has not been verified (Bauernfeind *et al*, 1987a). Interestingly, however, there is clear evidence that *H. influenzae* isolates responsible for colonisation of the lower respiratory tract, and pulmonary exacerbations in CF patients, are invariably non-capsulate. In some cases, it may be difficult to determine whether the strains isolated from respiratory secretions are the cause of infection or merely normal commensal flora of the upper respiratory tract. Respiratory exacerbations in CF patients caused by non-capsulate strains of *H. influenzae* are probably underdiagnosed and their pathogenic significance under estimated. Rayner (1990) showed that non-capsulate strains of *H. influenzae* are significant pathogens in children with CF as a rise in the number of organisms in sputum preceded the developement of acute exacerbation and clinical improvement coincided with a reduction in bacterial numbers after antimicrobial treatment. Studies on the use of C-reactive protein, as an indicator of pulmonary inflammation, also provided evidence that very high levels of the

inflammatory marker are associated with *H. influenzae* exacerbations in CF patients (Glass *et al*, 1988). Sputum cultures from CF patients show that *H. influenzae* can be isolated in similar numbers to those of *P. aeruginosa* suggesting both organisms play a significant role in the pathology of airway inflammation. Although *H. influenzae* is associated with pulmonary exacerbations in CF patients, there is no evidence to suggest it has a primary role in the chronic progressive pulmonary decline (Pressler *et al*, 1984).

The prevalence of *H. influenzae* in sputum culture from CF patients varies considerably, from 11% in a survey carried out in the USA (Gilligan, 1991) to 30% in Manchester (Bilton *et al*, 1995). This discrepancy in the rate of isolation may derive from differences in the methods involved in processing sputum specimens and on the age of patients investigated. Detection of *H. influenzae* in sputum cultures from CF patients can prove challenging especially when larger numbers of mucoid and non-mucoid *P. aeruginosa* are present in the specimen. Colonial morphology of *H. influenzae* may be difficult to distinguish from that of atypical non-mucoid colonial forms of *P. aeruginosa* associated with long-term pulmonary colonisation. The sheer mass of bacterial growth associated with mucoid *P. aeruginosa* can also suppress the growth (Moller *et al*, 1993) or even overgrow the smaller colonies of *H. influenzae*.

A number of methods have been proposed to increase the recovery rate of *H. influenzae* from CF sputum. The use of quantitative procedures in the processing

of sputum cultures as well as selective medium (eg. chocolate blood agar incorporating bacitracin) and incubation in an atmosphere of 5 to 10 % CO_2 may assist in overcoming this problem and account for the difference in the isolation rate between different studies. Roberts and Cole (1980) used a haemin-bacitracin medium incubated under anaerobic conditions and described an increase from 5 to 80% in the isolation rate. Bauernfeind *et al* (1987b) used pyocins to inhibit the growth of *P. aeruginosa* in order to enhance the recovery of *H. influenzae*. More recently, Moller *et al*, (1993) reported that the use of N-acetyl-D-glucosamine and cefsulodin discs in an anaerobic atmosphere improved the recovery rate of *H. influenzae*. Their rationale was that the lack of oxygen combined with the action of the antibiotic was inhibitory to *P. aeruginosa* whilst the presence of N-acetly-D-glucosamine allowed recovery of spheroplast forms of *Haemophilus*. The significance of *H. influenzae* in CF sputum culture, especially in small numbers, still remains debatable as it may be difficult to determine whether the organism is the cause of pulmonary infection or merely a contaminant from upper respiratory secretions.

6.5 EPIDEMIOLOGY

Biotyping and outer membrane protein analysis are the two most commonly used typing systems amongst many that are available for epidemiological studies of non-capsulate *H. influenzae*. However, these phenotypic systems are now being superceded by genomic methods. Biotyping of *H. influenzae* is a classification system based on enzymatic and biochemical properties of the organism. Initially,

H. influenzae was divided into five biotypes (I - V) on the basis of three tests involving ornithine decarboxylase, indole production and urea hydrolysis (Kilian 1976). Subsequently, an additional three biotypes, VI, VII and VIII were added (Table 6.1), also based on the same three tests (Oberhofer & Back, 1979, Gratten, 1983, Sottnek & Albritton 1984).

TABLE 6.1. Biotypes of *H. influenzae*

BIOTYPE	INDOLE	UREA	ODC
I	+	+	+
II	+	+	-
III	-	+	-
IV	-	+	+
V	+	-	+
VI	-	-	+
VII	+	-	-
VIII	-	-	-

ODC - ornithine decarboxylase
+ = positive result
- = negative result

There is little evidence that any particular biotype of *H. influenzae* is more pathogenic than any other; the exception is biotype I which is associated with enhanced virulence regardless of capsulation (Long *et al*, 1983) and is predominant in meningitis, septicaemia and otitis media in children. Biotype I is also responsible for 80% of infections in invasive disease with the majority of the strains being serotype b and capsulate (Long, 1983). The other biotypes are generally nonserotypeable. In a study of children with otitis media, 53% of the strains of

H. influenzae were biotype II (De Maria *et al*, 1984). Biotype II & III are predominant in respiratory infections in non-CF adults with chronic lung disease (Brabender *et al*, 1984) but are also found in the respiratory tracts of individuals with no evidence of respiratory infection (Watson *et al*, 1985). Biotype III has been shown to be the cause of septicaemia in USA (Kamme, 1980) and is found in both CF and non-CF adults with chronic lung disease (Bilton *et al*, 1995).

In patients with CF, *H. influenzae* has been found to be predominantly biotype I (Watson *et al*, 1985) although a subsequent study carried out by Rayner *et al* (1990) showed biotype V to be more common that biotype I. However, this study also demonstrated that biotype I was isolated more frequently immediately prior to an exacerbation. CF patients may harbour a particular biotype for several months but some show a change in biotype after an exacerbation (Rayner *et al*, 1990). Some patients may possess more than one strain of *H. influenzae*, although one strain tends to predominate, and up to four biotypes have been present simultaneously. This type of mixed infection differs from non-CF patients where there is rarely more than one biotype present at any one time (Watson *et al*, 1988).

Isolates of *H. influenzae* are commonly characterised by analysis of MOMP with SDS PAGE. This typing system is highly discriminatory with non-capsulate *H. influenzae* showing greater strain to strain variation compared to capsulate type b strains (Murphy *et al*, 1983). However, the MOMP patterns have been shown to change during persistent infection in patients with chronic obstructive pulmonary

disease (Groeneveld *et al*, 1988). Although SDS PAGE analysis of these *H. influenzae* variants showed variation in MOMP composition, genomic typing systems based on PCR and RFLP showed the isolates to be identical (Van Belkum *et al*, 1994). In studies with other *H. influenzae* isolates both genomic systems provided good strain discrimination with PCR having the advantage of producing results which are easier to interpret.

The surface of non-capsulate *H. influenzae* contains OMP and lipooligosaccharide (LOS), which exhibit antigenic heterogeneity and can be used to serotype isolates (Murphy & Apicella, 1987). Serological reactivity of OMP with specific antisera divides *H. influenzae* into six serotypes (Murphy & Apicella 1985). The structure of *H. influenzae* LOS, based on SDS-PAGE and silver staining, is fundamentaly different from that of LPS of enteric Gram negative bacilli (Flesher & Insel, 1978).

Multilocus enzyme electrophoresis (MLEE) is a means of characterising strains of *H. influenzae* based on the relative electrophoretic mobility of a number of different water-soluble metabolic enzymes. A drawback of this phenotypic typing system is that it analyses only a small fraction of the structural gene loci of *H. influenzae* in contrast to some of the genomic systems which examine the entire genome. MLEE was initially employed to estimate diversity of capsulate serotype b isolates of *H. influenzae* (Musser *et al*, 1985). A further study, again using MLEE, demonstrated a greater degree of diversity among non-capsulate isolates than was observed in capsulate serotype b isolates of *H. influenzae*. Interestingly, there was no

evidence of any sharing of MLEE types between capsulated and non-capsulated strains of *H. influenzae* (Musser *et al*, 1986).

In recent years, a number of genomic fingerprinting systems for *H. influenzae* have been developed. These include RNA gene restriction pattern analysis (Bruce & Jordens, 1991) which provides low discrimination; DNA - RFLP (Loos *et al*, 1989) which produces complex banding patterns and ERIC - PCR (Van Belkum, 1994) which is as discriminating as DNA-RFLP but provides more interpretable banding patterns. Jordens *et al* (1993) compared SDS-PAGE analysis of OMP, ribotyping and RAPD-PCR and showed concordance with all three methods.

6.6 AIMS

Biotyping has been frequently used for epidemiological investigations of *H. influenzae*, however, this phenotypic technique is now being superseded by genomic systems. PFGE, generally considered to be the gold standard of genomic systems, has not been investigated as a means of typing *H. influenzae* nor has the method been compared with the traditional phenotypic biotyping system.

Successful application of PFGE for bacterial fingerprinting relies to a great extent on a suitable method for extracting chromosomal DNA and optimising each of the running parameters: pulse times, run times and temperature of the running buffer. Adequate DNA digestion relies on finding a suitable rare cutting restriction enzyme.

This aim was addressed prior to the epidemiological aims outlined in the following paragraph.

As *H. influenzae* is present as normal human commensal flora in the upper respiratory tract, it can be difficult to determine whether organisms isolated from CF respiratory secretions are associated with infection or merely contaminants from the upper respiratory tract. Previous studies have also provided conflicting results as to which *H. influenzae* biotypes might be responsible for pulmonary exacerbations. One of the aims of this thesis was to examine *H. influenzae* isolates from both paediatric and adult CF patients to determine whether particular biotypes were present during pulmonary infection and whether there was a difference in the distribution of biotypes between children and adults. It was also proposed to examine *H. influenzae* isolates from non-CF patients to compare the biotypes found in CF and non-CF patients.

6.7 RESULTS

6.7.1 Developement of PFGE for *H. influenzae.*

Chromosomal DNA was prepared from *H. influenzae* by a modification of the method used by Vasil *et al* (1990), see Material and Methods. The next step was to find an appropriate restriction enzyme which would cleave genomic DNA from *H. influenzae*. Initially, *Xba*I was used as it successfully digested the genomes of the other Gram-negative bacterial species investigated. *Xba*I appeared to be successful in cutting *H. influenzae* DNA into 20 - 24 fragments. However, separation of the

fragments by PFGE resulted in profiles with banding patterns close together in the lower section of the agarose gel. In an attempt to rectify this problem, the effect of different pulse times on banding patterns was investigated. Optimum results were found with pulse times of 0.5s - 20s. Initially, agarose gel strength was 1% run at 200V in TBE buffer at 14^{o} C for 20h, but reduced later to 16h. Although these conditions slightly improved the PFGE profiles the bands were still confined to the lower section of the gel. Consequently, other restriction enzymes *Spe*I, *Sma*I and *Dra*I (TTTAAA) were investigated in the hope of achieving an enzyme capable of cleaving the DNA into fragments with a greater range of size distribution. The PFGE profiles produced using *Spe*I showed slightly better variation in DNA fragment sizes compared to those produced by *Xba*I; *Dra*I completely failed to digest DNA from *H. influenzae*. The best results were obtained using restriction enzyme *Sma*I which generated 12-19 fragments ranging in size from < 48.5 - 388 kb consequently, *Sma*I was selected as the enzyme of choice for the purpose of epidemiological studies. Figure 6.1 illustrates PFGE banding patterns of two isolates of *H. influenzae* using the restriction enzymes *Xba*I, *Spe*I, and *Sma*I.

In this thesis, the final conditions chosen for PFGE fingerprinting isolates of *H. influenzae* involved digestion with restriction enzyme *Sma*I and the following running parameters: initial time of 0.5s; final pulse time of 20s for 16h at 200V using TBE buffer at 14^{o} C. The diversity in PFGE profiles produced by 9 CF isolates of *H. influenzae* are shown in Figure 6.2.

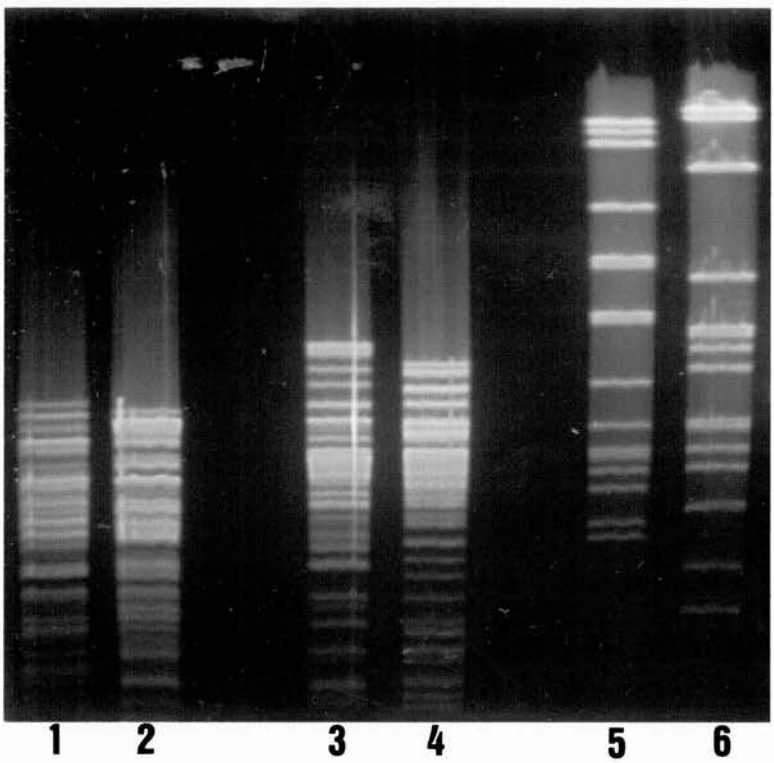


FIG. 6.1. PFGE profiles of two isolates of *H. influenzae* C2337 and C2386 digested with restriction enzymes *Xba*I in lanes 1 and 2; *Spe*I in lanes 3 and 4; *Sma*I in lanes 5 and 6, respectively.

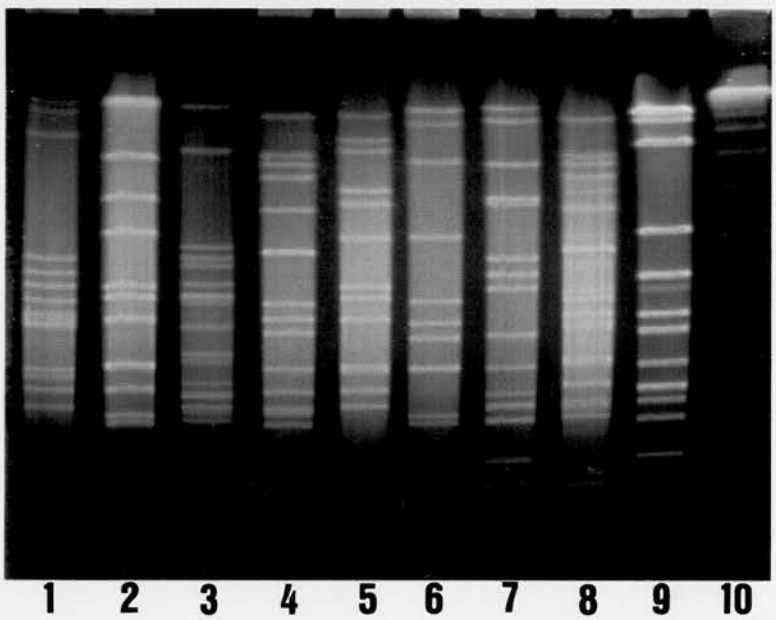


FIG. 6.2. *Sma*I digestion of DNA from nine CF isolates of *H. influenzae* showing the diversity of PFGE profiles. Lanes 1 to 9, C2491, C2481, C2827, C2830, C2831, C2849, C2851, C2737, C2832, respecitvely; lane 10, lambda concatemers (size range 48.5kb to 388kb, in increments of 48.5kb).

6.7.2 Comparison of Biotyping and PFGE

Preliminary studies comparing biotyping and PFGE were carried out on ten isolates of *H. influenzae* recovered from three adult CF patients undergoing CF gene therapy treatment. Biotyping showed that isolates from two of the patients were biotype II with the isolates from the third patient belonging to biotype V. On fingerprinting these isolates by PFGE, the biotype II isolates, from both patients, showed identical PFGE profiles while the isolates in biotype V were identical to each other but different from those produced by the biotype II strains.

Since these preliminary results suggested that there may be some correlation between the two typing systems a further 58 isolates of *H. influenzae* from 37 CF patients, comprising of 32 paediatric and 5 adults, were obtained from the Clinical Microbiology Department, Western General Hospital, Edinburgh. Thirty additional isolates of *H. influenzae*, recovered from sputum from non-CF hospitalised adults, were obtained from the Clinical Microbiology Department, Royal Infirmary, Edinburgh.

All 88 *H. influenzae* isolates were biotyped using conventional biochemical tests for indole, urea and ornithine decarboxylase. The isolates within each biotype were also genotyped by PFGE and the banding patterns examined to determine the relationship, if any, between isolates within a biotype. The results revealed that at least two isolates of *H. influenzae* within each of the biotypes I, II, III, and V produced similar or identical PFGE profiles; furthermore a strain common to both CF and non-CF

patients was observed in biotype V. All isolates in biotype IV and VI produced different PFGE patterns. There was no evidence of isolates from different biotypes producing the same PFGE profiles.

Analysis of biotyping results indicated that two of the 16 CF isolates of *H. influenzae* belonging to biotype I exhibited the same PFGE profile. Both isolates were obtained from sputum but from two different paediatric CF patients. None of the CF isolates in biotype II appeared to be related on the basis of PFGE, however, two non-CF isolates showed PFGE profiles, differing by only a single band. Four of the 11 CF isolates, in biotype III, produced similar PFGE profiles and these included two siblings C 2732 and C2698, whose isolates produced a two band difference (Fig. 6.3). One of the three CF isolates in biotype V showed similar PFGE banding to three of the non-CF isolates. No isolates, either from CF or non-CF patients, were found to belong to biotypes VII or VIII.

When multiple isolates of *H. influenzae* from 13 CF patients were typed by biotyping and PFGE the results obtained were concordant. All isolates in six of the patients appeared to be the same strain. However, biotyping and PFGE suggested that the remaining seven patients harboured two or more strains of *H. influenzae*. Figure 6.4. shows PFGE profiles from 3 CF patients colonised with 2 or 3 different strains of *H. influenzae* simultaneously.

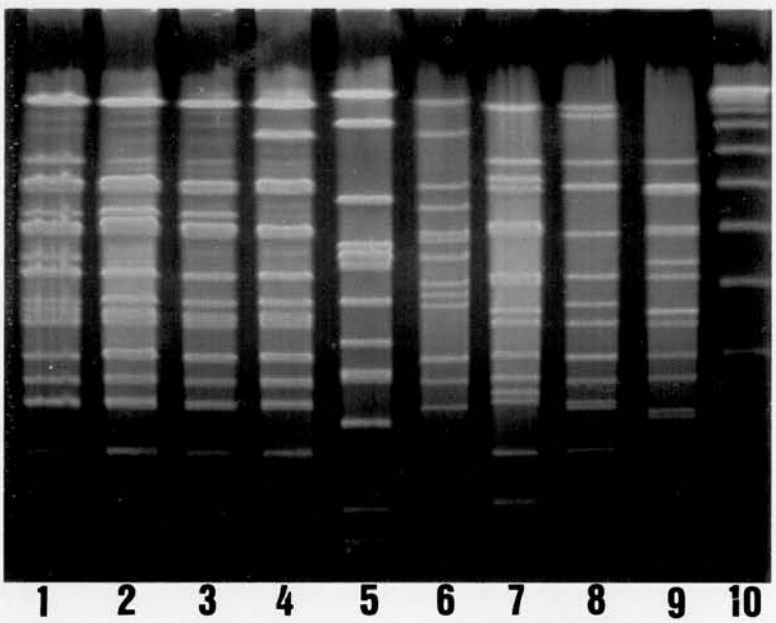


FIG. 6.3. *Sma*I digestion of DNA from isolates of *H. influenzae*, classified as biotype III. Lanes 1 to 7, CF isolates (C2688, C2699, C2732, C2698, C2547, 8399, 8328, respectively); lanes 8 and 9, non-CF isolates (6160 and 9841, respectively); lane 10, lambda concatemers (size range 48.5 kb to 388kb, in increments of 48.5kb).

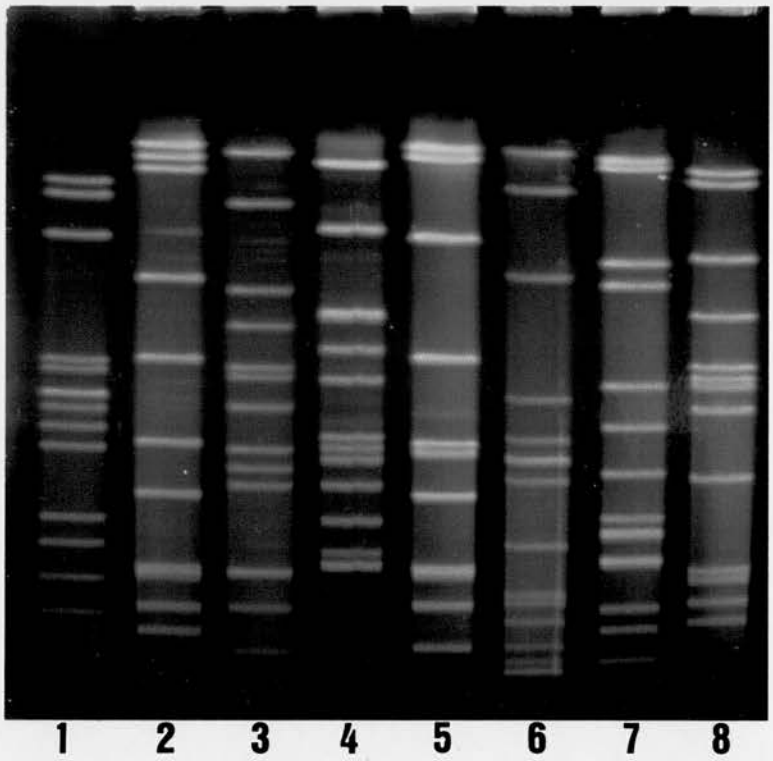


FIG. 6.4. *Sma*I digestion of isolates of *H. influenzae* from three CF patients, colonised with strains of different biotypes. Lane 1 to 3, (patient A C2687, C2656, 8399 - biotypes I, II and III, respectively); lanes 4 to 6, (patient B C2647, C2594, C2723 - biotypes I, II, and III, respectively); lanes 7 and 8, (patient C C2734, C2599 biotypes I and II, respectively).

6.7.3 Distribution of Biotypes

In calculating the prevalence of each biotype, only 45 isolates of *H. influenzae* from the 37 CF patients were included; patients with multiple isolates of the same biotype were only included once. The distribution of biotypes for both CF and non-CF isolates are shown in Table 6.2.

TABLE 6.2. Distribution of biotypes in CF and non-CF patients

BIOTYPE	CF (%)	SPECIMEN	NON - CF (%)
I	14 (31)	5 sputum (38%)	2 (7)
		9 T/S (28%)	
II	11 (24)	3 sputum (23%)	18 (60)
		8 T/S (25%)	
III	11 (24)	1 sputum (8%)	3 (10)
		10 T/S (31%)	
IV	4 (8)	1 sputum (8%)	2 (7)
		3 T/S (9%)	
V	3 (7)	1 sputum (8%)	5 (17)
		2 T/S (6%)	
VI	2 (4)	2 sputum (15%)	0

T/S, throat swab

Biotyping data demonstrated little difference in the incidence of biotype I, II, and III in CF isolates, with a slightly larger number of isolates falling into the biotype I group. Only a small number of CF isolates were found to be biotype IV, V or VI and no isolates were found in biotype VII or VIII. When the source of the isolate was taken into account, a difference in distribution of biotypes was observed. Thirty eight per cent of the isolates of *H. influenzae* recovered from sputum were biotype I compared with 23% biotype II, 15% biotype VI and only 8% in each of biotypes III,

IV, and V. In contrast, there was little difference in the distribution between biotypes I, II, and III in isolates recovered from throat swabs and the incidence of the other biotypes IV and V were similar to those found in the sputum isolates. No isolates of *H. influenzae* recovered from nose swabs were found to belong to biotype VI.

These results suggest a difference in the distribution of biotypes between CF and non-CF isolates ($p<0.01$, χ^2 test). The majority of isolates from non-CF patients belonged to biotype II with only small numbers in biotypes I, III, IV, and V, and no isolates in biotypes VI, VII, or VIII.

6.7.4 Can *P. aeruginosa* inhibit *H. influenzae*?

Problems in culturing *H. influenzae* from sputum culture are well documented, especially in patients who have been colonised with *P. aeruginosa* for a considerable time. Could this be an explanation as to why *H. influenzae* was only isolated from five adult CF patients? All 37 CF patients, from whom *H. influenzae* was recovered, were investigated to determine what other organisms were present in their sputum and to determine whether these might influence the recovery of *H. influenzae*. *P. aeruginosa* was isolated from 14 of the 32 paediatric patients and one patient was colonised with *B. cepacia*. Of the five adults, four were colonised with both *P. aeruginosa* and *B. cepacia* and the fifth patient with *B. cepacia* alone. Isolates of *H. influenzae*, *P. aeruginosa* and/or *B. cepacia*, from the same patient, were tested against one another to see if they showed any signs of an inhibitory affect. Interestingly, in all cases *P. aeruginosa* inhibited the growth of *H. influenzae*,

regardless of whether the strain was mucoid or non-mucoid whereas *B. cepacia* was not inhibitory to any of the isolates of *H. influenzae*. An additional six strains of *P. aeruginosa*, isolated from other CF patients, were checked for inhibition against a selection of 12 different strains of *H. influenzae*. In all cases, *P. aeruginosa* inhibited the growth of *H. influenzae* in all cases.

6.8 DISCUSSION

RFLP analysis following whole DNA digestion and PFGE appears to be a valuable and discriminatory technique for characterisation of *H. influenzae* strains. Unlike some other organisms, the PFGE profiles produced by *H. influenzae* were always clear, sharp and easy to interpret. A frequent comment of PFGE is that it is time-consuming. In fact, it is no more time consuming than conventional, non-commercial, biochemical analysis. However, if commercially available kits are used for biotyping *H. influenzae*, results are available within 24 h - 48 h; in contrast PFGE takes at least three days.

Comparative analysis of biotyping and PFGE confirmed the preliminary findings and indicated a degree of concordance between the two typing systems. Within biotypes I, II, III, and V there were at least two isolates of *H. influenzae* with the same PFGE profile and no isolates with different biotypes were found to have the same PFGE profile. However as expected, PFGE proved to be more discriminatory than biotyping.

An interesting difference was observed in the distribution of biotypes between CF and non-CF patients with the majority of isolates of *H. influenzae* in the non-CF group belonging to biotype II. These results are similar to those observed by Brabender *et al* (1984) who found that biotypes II and III were predominantly responsible for respiratory infection in non-CF adults.

As mentioned previously, it can prove difficult to judge whether an isolate of *H. influenzae* recovered from a CF patient is a pathogen or merely commensal flora from the throat. However, the presence of *H. influenzae* in sputum culture at levels of $>10^6$ orgs/ml generally indicates that this organism should be considered to be the cause of, or contributed to, pulmonary infection. In such cases, the predominant biotype was biotype I. Further studies are required to determine whether isolates of biotype I are associated with pulmonary infection. Two previous studies have shown that biotype I was predominant in CF patients (Watson *et al*, 1985) although in one study biotype I isolates were only predominant immediately prior to an exacerbation. In most cases biotype V was the predominant biotype (Rayner *et al*, 1990). In studies for this thesis, only three *H. influenzae* isolates were found to belong to biotype V. Interestingly, all five isolates of *H. influenzae* from adult CF patients belonged to different biotypes.

H. influenzae is not a common cause of pulmonary exacerbation infection in adult CF patients and subsequently only a small number of isolates were obtained from this group of patients. This study also demonstrated that *P. aeruginosa* has the ability to

inhibit the growth of *H. influenzae*, certainly *in vitro*. As the majority of CF adults are colonised with *P. aeruginosa*, could this account for the low incidence of *H. influenzae* recovered from sputum culture in this group of patients? However, this explanation does not explain why 14 of the 32 paediatric CF patients were colonised with *P. aeruginosa* together with 4 of the 5 adult patients.

CHAPTER 7

PSEUDOMONAS AERUGINOSA

7.1 TAXONOMY AND NOMENCLATURE

Fordos (1860) described the pigment responsible for the blue-green appearance of infected wound pus, named it pyocyanine, and reported it to be soluble in water, alcohol, ether and chloroform. It was not until 1872, however, that Schroeter stated that a motile microbe was responsible for the production of this pigment. Despite neither chemical or microscopic observation for this theory, he proposed the name *Bacterium aeruginosum* for this bacterium. Ten years later, Gessard (1882) isolated and described the organism which produced pyocyanin but did not propose a scientific name for the organism. However, the name *Bacillus pyocyaneus* was attributed to Gessard by a number of authors. The generic name *Pseudomonas* (Greek for 'false unit') was first used by Migula in 1894 and the species name *pyocyanea* (Greek for 'blue pus'). Subsequently, Migula altered the species epithet in 1900 to *P. aeruginosa* (Latin, ' full of copper rust or verdigris', hence green). Controversy continued as to whether the species should be called *pyocyanea* or *aeruginosa* until the Judical Commission of the International Committee on Nomenclature of Bacteria (1952) stated that *P. aeruginosa* was the name of choice. Finally, in 1994 the species epithet *aeruginosa* was accepted as the official denomination of the bacterium in Bergey's Manual of Determinative Bacteriology (Holt *et al*, 1994)

7. 2 GENERAL CHARACTERISTICS

P. aeruginosa is a Gram-negative non-sporing bacillus, motile, usually with a single polar flagellum and polar fimbriae. This organism is a strict aerobe although nitrate can be used as an electron acceptor to permit growth under anaerobic conditions. Growth occurs over a wide range of temperatures, 4 - 42^{o}C, with an optimum of 37^{o}C. Unlike most pseudomonads, *P. aeruginosa* will grow at 42^{o}C but is killed by exposure at 55^{o}C for 1 hour.

P. aeruginosa can produce six distinct colonial morphotypes, designated 1-6 with type 1 the classic, pigmented non-mucoid morphotype being most common (Phillips, 1969). Type 2 colonies are small, smooth and coliform-like; types 3 & 4 are small, rough and rugose; type 5 describes the mucoid morphotype associated with overexpression of alginate exopolysaccharide. Type 6 are dwarf colonies; these may appear slightly mucoid as they are usually variants of the mucoid type. All six types have been found in sputum specimens from CF patients.

Non-pigmented or atypical strains of *P. aeruginosa* can prove difficult to identify and in such cases identification relies on the use of biochemical tests, in particular the characteristic cytochrome oxidase test with the majority of strains producing a positive reaction. *P. aeruginosa* has the ability to oxidise a number of carbohydrates with acid production only. Other biochemical tests include indole, hydrogen sulphide, Voges Proskauer, methyl red, lysine decarboxylase and aesculin which

produce negative reactions whereas arginine dihydrolase, catalase and gelatin liquifaction are positive. A variety of extracellular enzymes are also produced by *P. aeruginosa* including proteases, lipases, esterases and elastases.

P. aeruginosa can grow readily on minimal medium and use a wide range of organic substances as carbon and nitrogen sources. The use of selective medium incorporating selective agents, including Dettol, cetrimide or acetamide, may assist in the isolation and identification of *P. aeruginosa*. *P. aeruginosa* can be recovered from a variety of sources ranging from soil, vegetation, surface water, plants and animals; it is also a commensal in the intestinal and respiratory tracts of a small proportion of healthy humans. *P. aeruginosa* is a classic opportunistic pathogen with innate resistance to many antibiotics and disinfectants and has the ability to cause a wide range of infections in patients compromised by underlying disease or treatment. It grows as a saprophyte in warm, moist situations and is commonly found in hospital environments, including sinks, drains, humidifiers, respirators and disinfectant solutions. Swimming pools and jaccuzis may also act as reservoirs of *P. aeruginosa*, particularly if disinfection is impaired. Two main characteristics of *P. aeruginosa* contribute to this ubiquity; first, the ability to use more than 50 organic compounds as energy and/or carbon sources (Stanier *et al*, 1966), second, a capacity to survive lengthy periods of time in moist environments. *P. aeruginosa* does not only survive but utilises diesel fuels and kerosene as carbon sources for growth (Bushnell & Hass, 1941) and has been shown to grow in distilled water (Favero *et al*, 1971). Like many Gram-negative bacteria, *P. aeruginosa* is relatively sensitive to drying.

The four distinct pigments produced by *P. aeruginosa* are pyocyanin, pyoverdin, pyorubrin and pyomelanin. The characteristic blue phenazine pigment, pyocyanin, which is soluble in both chloroform and water, is only found in *P. aeruginosa* and is produced by approximately 80% of strains. Production of pyocyanin can be enhanced with the addition of glycerol, magnesium, potassium or iron to the culture media. Pyocyanin acts as a potential antibacterial agent and has a bactericidal affect on a number of bacterial species, notably *Escherichia coli*, *Staph. aureus* and *Mycobacterium smegmatis* (Schoental, 1941). Pyoverdin is a yellow/green pigment (fluorescein) produced by most strains of *P. aeruginosa* and other fluorescent pseudomonads. This pigment, which is an important iron-chelating siderophore, is formed in the presence of phosphate and low iron and is soluble in water but not chloroform. Colonies producing pyoverdin will fluoresce under UV light. Pyorubrin and pyomelanin are less commonly found pigments. Pyorubrin produces a reddish pigmentation, which is enhanced by the addition of 1% DL-glutamate to the culture medium, and pyomelanin produces a brownish-black pigmentation and may require the addition of 1% tyrosine to culture medium.

In some *in vitro* and *in vivo* environments, *P. aeruginosa* grows as microcolonies forming an adherent matrix or biofilm which can be observed by light and electron microscopy (Lam *et al*, 1980). This biofilm mode may be an important factor in the organism's universal distribution in natural environments. Biofilms offer a survival advantage to a bacterium as the inner cells are protected by an outer layer of exterior

cells and exopolysaccharide which also protects the organisms from host immunity and antimicrobial treatment.

7.3 PATHOGENICITY

P. aeruginosa seldom causes infection in healthy individuals but is one of the most common pathogens involved in nosocomial infections; individuals particularly susceptible are immunocompromised patients and intubated patients in intensive care units, or patients predisposed to infections as a result of destruction of physical barriers such as in burns, eye damage and surgery (Dart & Seal, 1988). Infections include septicaemia, urinary tract, ear infections (including 'occupational' infections in deep sea divers), corneal ulceration due to contamination of contact lens fluid and respiratory tract infections in patients with CF, COPD, AIDs and cancer (Alcock, 1976; Speert, 1994; Zloty & Belin, 1994; Spencer, 1996). Although *P. aeruginosa* is a serious cause of hospital infection, the highest morbidity and mortality occurs in individuals with underlying diseases such as CF and cancer. The pathogenesis of infections caused by *P. aeruginosa* is multifactorial and complex with different virulence factors exhibiting different levels of importance. *P. aeruginosa* produces a considerable armoury of virulence factors which include fimbriae, flagella, lipopolysaccharide (LPS), slime, alginate, exotoxin A, exoenzyme S, haemolysins, proteases, elastase, cytotoxin, siderophores and pyocyanin.

Most strains of *P. aeruginosa* produce polar fimbriae or pili, which are associated

with adherence of the organism to various cell surfaces. *P. aeruginosa* pilin adheres to cell surfaces via the receptor site, GalNAcβ1-4Gal, which is present in certain asialylated glycoproteins, including asialoganglioside GM1 (aGM1) (Krivan *et al*, 1988). However, these binding sites are not specific to *P. aeruginosa* as other bacterial pathogens including *Staph. aureus* also adhere readily to aGM1 (Imundo *et al*, 1995).

The single polar flagellum of *P. aeruginosa* is an important taxonomic characteristic of this organism. Flagella are proteinaceous, filamentous cell surface appendages responsible for motility and chemotaxis of the organism. They are associated with virulence as non-flagellate strains are less likely to cause systemic infections in the burned mouse model than their flagellate counterparts (Drake & Montie, 1988).

The LPS of *P. aeruginosa* comprises lipid A, which is integrated into the bacterial outer membrane, and a core region which extends from lipid A. In smooth strains, a carbohydrate side chain, known as the O-specific side chain, is attached to the core region. The composition and structure of the side chains are variable, forming the basis of serotyping, whereas the lipid A and core region are relatively conserved. LPS plays a major role in the virulence of *P. aeruginosa* and other Gram-negative organisms conferring a resistant barrier to the bactericidal action of complement in human serum and protection of the bacterial cell against opsonisation and phagocytosis (Engels *et al*, 1985). Most clinical isolates of *P. aeruginosa* are

smooth, indicating the possession of O-antigen and are resistant to normal human serum. In contrast, isolates from chronic infections, including those involving the respiratory and urinary tracts are deficient in O-side chains and are serum sensitive (Hancock *et al*, 1983). However, there is no absolute relationship between serum resistance and possession of O antigenic side chains. The LPS of *P. aeruginosa* is similar in general structure to that of *Enterobacteriacae* but has a lower intrinsic toxicity (Pitt, 1988; Liu, 1974). Unlike enterobacteria, serologically rough strains of *P. aeruginosa* are seldom 'rough' in colonial appearance (Pitt, 1988). There is little doubt that LPS plays a significant role in *P. aeruginosa* infections as antibody towards LPS is highly protective in human infections and in animal models (Pollack & Young, 1979).

All strains of *P. aeruginosa* produce extracellular slime which is distinct from the alginate-like exopolysaccharide associated with the mucoid colonial morphotype. The composition of pseudomonas slime varies depending on the strain, cultural conditions and method of analysis (Govan, 1990). More than 50% of the slime is composed of polysaccharide, mainly glucose and mannose with around 20% nucleic acid and a small protein fraction (Brown *et al*, 1969).

The most toxic virulence factor of *P.aeruginosa* is exotoxin A, an exoenzyme produced by more than 90% of strains (Vasil, 1986). Exotoxin S is more heat resistant than exotoxin A and is partially destroyed by the reducing agents urea and dithiothreitol which interestingly increase the activity of exotoxin A. Exotoxin S

may act as an adhesion factor and play an important role in the initial adherence of *P.aeruginosa* to epithelial cells (Baker *et al*, 1991).

Most *P.aeruginosa* produce phospholipase C and rhamnolipid (Vasil, 1986). Phospholipase C is a heat-labile haemolysin; rhamnolipid is heat-stable and inhibits mucociliary clearance.

Most *P. aeruginosa* produce a range of proteases which are active against a variety of substrates, including gelatin, casein, elastin and fibrin (Liu, 1974). Elastase is produced by 85-90% of strains and is active against human lung elastin, immunoglobulins, complement factors, human collagens and human mucins. Clinical isolates of *P. aeruginosa* produce significant levels of haemolysin, proteases, gelatinase, fibrinolysin, lipase, coagulase, lecithinase and DNase in contrast to environmental isolates which are relatively inert enzymatically. However, Mansi *et al* (1995) reported variability in expression of virulence factors in both clinical and environmental strains of *P. aeruginosa*.

Leucocidin is produced by most strains of *P.aeruginosa* and is an inactive or weakly active toxin capable of conversion to an active toxin by the action of various proteases, including elastase. Thus, leucocidin may contribute to inflammation and human tissue necrosis indirectly following release of proteolytic enzymes from bacterial and host cells.

7.4 *P. AERUGINOSA* IN CF PATIENTS

P. aeruginosa is the most common and life-threatening pathogen in CF with a prevalence reaching 80% or higher in CF adults (Pier, 1985; Gilligan, 1991; Govan & Deretic, 1996). Characteristically, pulmonary colonisation with *P. aeruginosa* increases with age (Govan *et al*, 1987; Gilligan, 1991) and tends to appear in early adolescence following previous infections with *Staph. aureus* and *H. influenzae*. The association of *P. aeruginosa* with the CF lung is complex and involves a range of host/pathogen interactions (Hutchison & Govan, 1999) which will be described in the following sections and summarised diagramatically in Fig.7.1a.

7.4.1 Aquisition of *P. aeruginosa*

Several theories have been proposed over the years to identify possible *P. aeruginosa* reservoirs and explain the mechanism of *P. aeruginosa* colonisation in CF patients. Apart from natural sources such as soil and surface water, the gastrointestinal tract has also been investigated as a possible source of non-mucoid *P. aeruginosa*; however the organism was only isolated from the faeces in CF patients who already had *P. aeruginosa* present in their sputum (Agnarsson *et al*, 1989). It was therefore difficult to determine whether gut colonisation preceded or resulted from colonisation of the respiratory tract. Finally, contamination of the gut from the respiratory tract was confirmed by Speert *et al* (1993) who demonstrated that respiratory colonisation preceded recovery from the gastrointestinal tract.

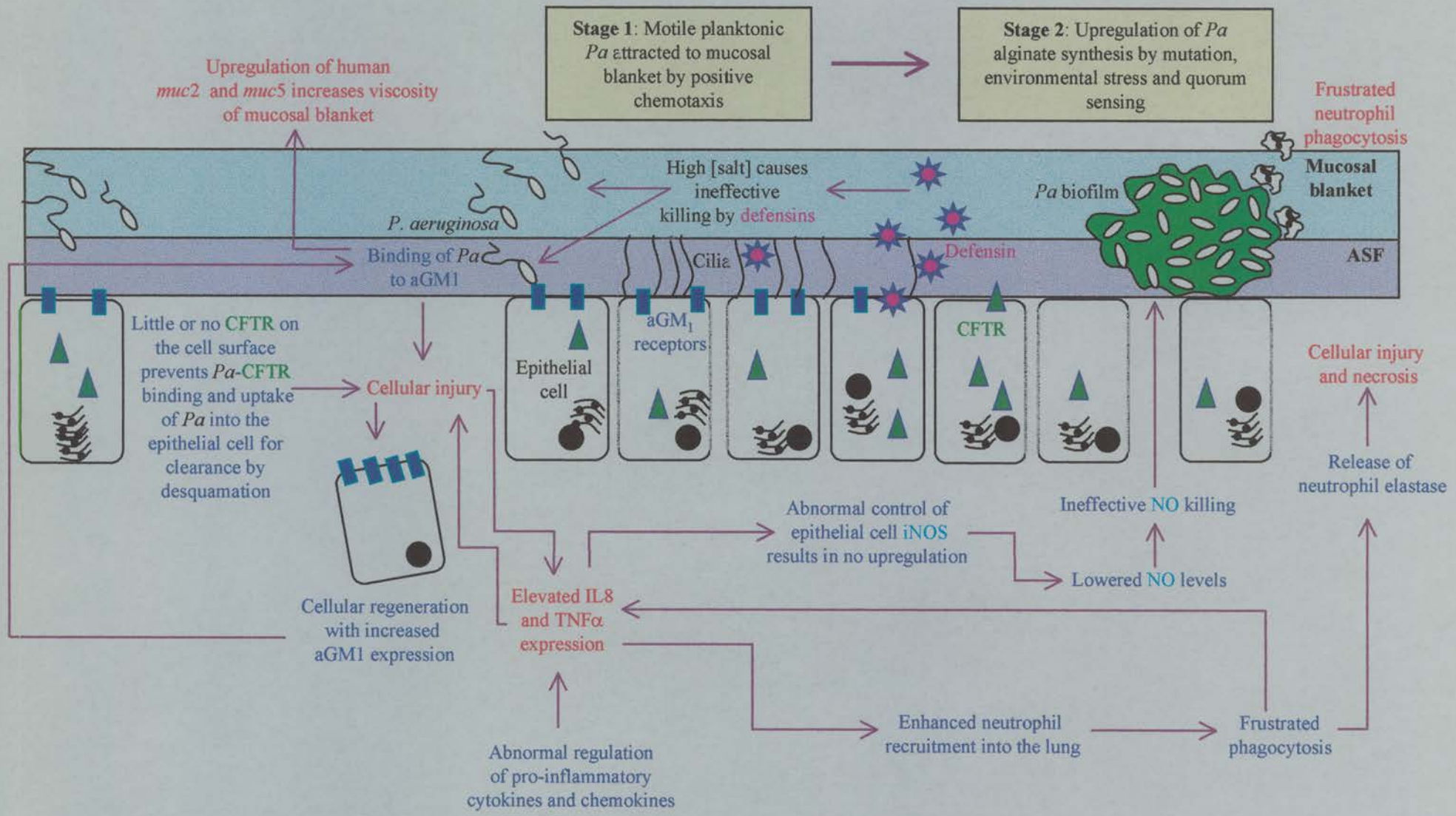

Stage 1: Motile planktonic Pa attracted to mucosal blanket by positive chemotaxis
Stage 2: Upregulation of Pa alginate synthesis by mutation, environmental stress and quorum sensing
Upregulation of human muc2 and muc5 increases viscosity of mucosal blanket
Frustrated neutrophil phagocytosis
Mucosal blanket
ASF
Pa biofilm
High [salt] causes ineffective killing by defensins
P. aeruginosa
Binding of Pa to aGM1
Cilia
Defensin
Little or no CFTR on the cell surface prevents Pa-CFTR binding and uptake of Pa into the epithelial cell for clearance by desquamation
Cellular injury
Epithelial cell
aGM1 receptors
CFTR
Cellular injury and necrosis
Release of neutrophil elastase
Abnormal control of epithelial cell iNOS results in no upregulation
Ineffective NO killing
Lowered NO levels
Cellular regeneration with increased aGM1 expression
Elevated IL8 and TNFα expression
Abnormal regulation of pro-inflammatory cytokines and chemokines
Enhanced neutrophil recruitment into the lung
Frustrated phagocytosis

FIG.7.1a

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FIG. 7.1a. Effects of CFTR dysfunction on pulmonary immunity and the implications for *P. aeruginosa* colonisation. Stage 1: Attracted by positive chemotaxis to host mucin, *P. aeruginosa* binds to the aGM1 receptor on the apical surface of the lung epithelium. CFTR dysfunction causes epithelial upregulation of IL-8, a potent neutrophil attractant, and a larger than appropriate neutrophil response. Neutrophil elastase and reactive species, as well as virulence factors exported by *P. aeruginosa* contribute to damage of the pulmonary epithelium raising further levels of pro-inflammatory cytokines and recruiting more neutrophils to the CF lung. Damaged epithelial cells regenerate with increased apical expression of aGM1 which allows more *P. aeruginosa* to bind to the epithelial cell surface. Thus an upwardly spiralling cycle of host damage and enhanced pathogen binding is initiated. Binding of *P. aeruginosa* LPS to aGM1 results in upregulation of human *muc* genes thereby increasing the burden of an already viscid mucosal blanket. A higher than normal concentration of salt in the airway surface fluid (ASF) of CF patients is believed to cause diminished activity of antimicrobial defensins. As a consequence, the natural antimicrobial activity of the ASF is compromised and fails to control the ever-increasing numbers of *P. aeruginosa*. Furthermore, aberrant control mechanisms fail to react to increased levels of pro-inflammatory cytokines and hence do not upregulate levels of the inducible nitric oxide (NO) synthetase enzyme in the pulmonary epithelia. Thus, the levels of the highly reactive antimicrobal NO molecule are lower than would be otherwise expected and the antimicrobial properties of the ASF are further diminished, promoting *P. aeruginosa* proliferation. Other proposed mechanisms that contribute to the lowered lung defences include the

CFTR desquamation hypothesis. The majority of CF patients lack apical CFTR, whose properties include specific *P. aeruginosa* binding. Since binding of *P. aeruginosa* to CFTR is thought to result in uptake of the pathogen into the epithelium, followed by clearance by desquamation, the lack of apical CFTR in CF compromises another pulmonary defence mechanism. Stage 2: As numbers of *P. aeruginosa* rise, a combination of spontaneous mutation, quorum sensing and environmental stress causes upregulation of bacterial alginate and formation of a bacterial biofilm. Key properties of the biofilm are decreased susceptibility to antibiotic and frustrated phagocytosis leading to neutrophil degranulation and inflammatory damage (Hutchison & Govan, 1999).

7.4.2 Adherence

Following flagella-mediated chemotaxis to the mucociliary blanket, adherence of *P. aeruginosa* to respiratory epithelium and respiratory mucus is thought to play a crucial role in the initial colonisation of the CF airways (Woods *et al*, 1980). Interestingly, it has also been suggested that flagella are important in the colonisation since they may act as a tether in initial interaction with epithelial membranes. This function, however, is offset by the contribution of flagella to phagocytic clearance (Feldman *et al*, 1998). It has been suggested that *P. aeruginosa* adhere more avidly to buccal epithelial cells of CF patients compared with normal individuals. This attachment is thought to be due to the action of dietary pancreatic supplements which strip fibronectin from the buccal cell surface allowing attachment of *P. aeruginosa* (Woods *et al,* 1980). *P. aeruginosa* is believed to adhere to epithelia primarily by

means of pili (Fick *et al*, 1992; Prince, 1992) but exoenzyme S (Baker *et al*, 1991), mucin and alginate (Pedersen, 1992) also have adhesin properties. Mucin is a major component of respiratory mucus together with other defensins lactoferrin, immunoglobulins, lipids, lysozyme and serum glycoproteins, some of which may have antimicrobial functions (Vishwanath & Ramphal, 1984). Mucus and cilia are the key components involved in mucociliary clearance. However, in CF patients clearance is impaired by the dehydration of the mucosal blanket and as a consequence *P. aeruginosa* adherence to mucin probably plays an important step in the early colonisation of the respiratory tract. Subinhibitory concentrations of ceftazidime inhibit the binding of mucoid *P. aeruginosa* strains to tracheobronchial mucins whereas tobramycin has no effect (Vishwanath *et al*, 1987).

Non-mucoid strains of *P. aeruginosa* do not adhere well to normal ciliated epithelial cells indicating that prior damage of the epithelia by viral or bacterial infections may be necessary for colonisation to occur (Ramphal & Pyle, 1983). As mentioned previously, *P. aeruginosa* pilin adheres to epithelial cells via the receptor site, GalNAcβ1-4Gal. Since these sites are not generally exposed, a number of factors have been considered to explain the increased availability of aGM1 receptor sites in the CF lung (Krivan *et al*, 1988). It has been suggested that the *P. aeruginosa* exoproduct, neuraminidase, increases the availability of aGM1 receptors by cleaving terminal sialic acid residues from cell surface gangliosides which may contribute to the initial bacterial colonisation process in the respiratory tract (Cacalano *et al*, 1992).

Defective CFTR may also play a role in the colonisation of CF airways with *P. aeruginosa*. Several studies have shown that CFTR dysfunction leads to an increase in aGM1 on the apical surface of airway epithelial cells (Saiman & Prince, 1993; Immundo *et al*, 1995; Davies *et al*, 1997). Furthermore, De Bentzmann and colleagues have demonstrated enhanced aGM1 receptors on regenerating epithelial cells, causing increased adhesion of *P. aeruginosa* (De Bentzmann *et al*, 1996). This is an interesting result since increased a GM1 would explain why *P. aeruginosa* tends to follow *Staph. aureus* in childhood (Gilligan, 1991) and the association of *P. aeruginosa* with previous infection by RSV (Petersen *et al*,1981).

7.4.3 Mucoid conversion

P. aeruginosa isolates cultured from initial pulmonary infection in CF patients are typically pigmented and non-mucoid and indistinguishable from *P. aeruginosa* isolated from non-CF infections. However, once *P. aeruginosa* becomes established in CF airways the organism undergoes a number of highly characteristic phenotypic changes associated with adaptation to the lung environment and evasion of host immunity. The most striking change is the production of alginate resulting in a mucoid colonial morphotype which is rarely seen in non-CF patients with the exception of patients with COPD (Doggett *et al*, 1966; Hoiby, 1975; Govan & Deretic, 1996).

Mucoid variants of *P. aeruginosa*, belonging to Phillips colonial morphotype 5, produce large amounts of the unusual non-capsular exopolysaccharide, alginate,

which is chemically distinct from pseudomonas slime. The association of mucoid *P. aeruginosa* with CF was first made by Doggett and colleagues in the 1960's (Doggett *et al*, 1964; Doggett *et al*, 1966; Doggett, 1969) and the exopolysaccharide identified as alginate by Linker & Jones (1964). Alginate produced by mucoid *P. aeruginosa* is a strongly anionic polysaccharide composed of acetylated D-mannuronic acid and L-guluronic acid (Evans & Linker, 1973). Govan & Harris (1986) have shown that mucins, released from respiratory epithelium following the action of pseudomonas proteases, combine with alginate, in the presence of divalent cations, particularly Ca^{++}, to form highly viscous gels. Gel formation enhances microcolony formation which may result in poorer mucociliary clearance of the organism and 'frustrated' phagocytosis leading to release of tissue damaging neutrophil elastase. Alginate production is not restricted to the mucoid colonial phenotype as non-mucoid revertants of mucoid *P. aeruginosa* have been found to produce alginate albeit at very much lower levels than in mucoid forms (Pier *et al*, 1986). Mucoid *P. aeruginosa* are so characteristic of CF lung infections that their culture from sputum is almost diagnosis of CF *per se*. However, mucoid variants are also responsible for approximately 40% of *P. aeruginosa* cultured from cases of COPD and 10% from urinary tract infections (McAvoy *et al*, 1989). Examination of post mortem lung material from CF patients infected with *P. aeruginosa* shows the bacteria in the form of distinct fibre-enclosed microcolonies in the infected airways (Lam *et al*, 1980). These authors speculated that growth of mucoid *P. aeruginosa* in microcolonies and biofilms in the CF lung plays an important role in pathogenesis by protecting the bacteria from surrounding environmental factors such as complement

and antibiotics. Alginate production is not exclusive to *P. aeruginosa* as it is also produced by strains of *Azotobacter vinelandii* (Gorin & Spencer, 1966) and other pseudomonads including *P. fluorescens, P. putida* and *P. mendocina* (Govan *et al*, 1981).

In CF patients, the time interval for conversion from the classic non-mucoid to the mucoid phenotype of *P. aeruginosa* varies but may be as short as three months (Govan, 1990). As infection becomes chronic, many CF patients harbour both mucoid and non-mucoid strains of *P. aeruginosa* concurrently; however, some CF patients reach adulthood without becoming chronically colonised or infected with mucoid *P. aeruginosa*. Long term colonisation with mucoid *P. aeruginosa* may damage the lung in two ways, firstly, tissue damage due to the *in situ* production of virulence factors such as elastase, exoenzyme S and exotoxin A and secondly, the constant aggrevation of the inflammatory response leading to neutrophil degranulation and immune-mediated damage. The alginate exopolysaccharide produced by mucoid *P. aeruginosa* also associates with host mucin and DNA forming bronchial plugs (Fig. 7.1b) which impair ciliary clearance and assist *P. aeruginosa* to avoid phagocytosis and other antimicrobial agents (Govan & Nelson, 1992).

FIG. 7.1b Plugged bronchial airway during chronic colonisation with mucoid *P. aeruginosa* (Govan, 1989).

The conversion of non-mucoid *P. aeruginosa* to the mucoid morphotype is controlled by a number of bacterial genes. In non-mucoid *P. aeruginosa*, the protein AlgU (product of the *alg*U gene) which plays an important role in transcription of alginate biosynthetic genes is repressed by Muc A and possibly MucB (products of *muc*A and *muc*B genes, respectively). In mucoid strains, mutations that inactivate the *muc*A gene relieve AlgU from inhibition which in turn activates *alg*D, a key

alginate biosynthetic gene, and thus alginate production is achieved (Deretic *et al*, 1993; Govan & Dereetic, 1996). It is thought that *P. aeruginosa* mutants with inactivated *muc*A are selected in the CF lung and that these alginate producers establish a biofilm in which there is effective protection against opsonisation, phagocytosis and antibiotic treatment.

The protective properties of alginate for *P. aeruginosa* include defence against the action of complement (Pedersen *et al*, 1990) and damaging free radicals. Environmental conditions which influence *P. aeruginosa* alginate production and selection *in vitro* include nutrient limitation (Speert *et al*, 1990), increased osmolarity (Govan, 1990), response to stress (Deretic & Govan, 1996), selection by phage (Govan, 1975) and antimicrobial pressure (Govan, 1976).

7.4.4 LPS deficiency

Many isolates of *P. aeruginosa* from CF patients have altered LPS with loss of all or part of the polysaccharide O-antigenic side chain (Hancock *et al*, 1983). This smooth to rough transition results in only a small percentage of strains from CF patients being serotypeable, the majority of strains being untypeable, polyagglutinable or autoagglutinable with O antisera. LPS O-antigen deficient or rough forms are invariably serum sensitive, *i.e.* killed by human serum while smooth or LPS complete strains are serum resistant, *i.e.* protected against complement-mediated killing by normal human serum. There is, however, no absolute relationship between serum susceptibility and possession of O-antigenic side chains. There is also no

correlation between mucoidy and serum sensitivity, typeability, LPS phenotype or OMP patterns of CF isolates. The property of serum sensitivity which seems to be associated with the LPS phenotype may explain why patients with CF rarely suffer from bacteraemia due to *P. aeruginosa* (Hancock *et al*, 1983).

7.4.5 Serum antibody response

Antibodies are produced in response to initial colonisation with *P. aeruginosa* in CF patients and an increased humoral host response in some patients is associated with poor prognosis (Hoiby, 1993). IgA and IgG antibodies are significantly elevated in CF compared to normal individuals but IgM is not (Schwartz, 1966). These findings were confirmed by Turner *et al* (1978) although the increase in IgA and IgG was only observed during exacerbations. Precipitating antibodies against *P. aeruginosa* are commonly detected in CF patients and are always detectable when the strains are mucoid (Burns & May, 1968). Antibodies against *P. aeruginosa* may prevent septicaemia but are unable to eliminate the bacteria from the lungs of CF patients (Pedersen *et al*, 1989); paradoxically, patients with high titres of antibodies are those who are commonly chronically colonised by this organism.

7.4.6 Antibiotics

It is often said that antibiotic treatment of *P. aeruginosa* infection in CF patients seldom if ever eradiates the organism. This is not strictly true. Treatment of non-mucoid *P. aeruginosa* in the early stages can result in eradication (Govan *et al*, 1987) and during the last decade two important studies have shown that aggressive early

treatment can eradicate *P. aeruginosa.* However, this is usually only a temporary phenomena as the organisms return at a later date with the interval of time for re-colonisation varying with each patient. In most patients, it becomes increasingly difficult to eradicate the organisms on subsequent infection and eventually a stage is reached when the organisms remain (regardless of whether they are sensitive to the antibiotic used) and the patients become chronically colonised. Littlewood and colleagues (1985) showed that treatment, of young CF patients with nebulised colomycin as soon as *P. aeruginosa* was isolated from throat swab or sputum delayed chronic colonisation. This aggressive early treatment reduced the number of organisms present in CF secretions and also the frequency of isolation of *P. aeruginosa*. A controlled study of Danish CF patients treated with ciprofloxacin and colomycin and untreated patients showed that 58% of the untreated patients became chronically colonised with *P. aeruginosa* while only 14% of treated patients became chronically colonised (Valerius *et al*, 1991). These Danish results confirm the findings of Littlewood and colleagues (1985) that early treatment with antibiotics helps to prevent or delay colonisation with *P. aeruginosa*.

The present Danish approach is to administer intensive therapy, with a combination of intravenous and nebulised antibiotics, at regular three month intervals regardless of whether the patient has an exacerbation or not. This is claimed to increase survival rates even after colonisation has occured (Hoiby, 1993). The Danish strategy is not universally accepted and has problems of cost, side-effects of antibiotics and development of resistance.

CF patients are generally treated with two antibiotics to reduce the risk of the emergence of resistant strains; this is usually a combination of an aminoglycoside and a β-lactam, third generation cephalosporin or a carbapenem (Pitt, 1986; Livermore, 1989). A particular problem in the use of antibiotics for infections in CF patients is the difficulty of achieving an adequate therapeutic dose of antibiotic at the target site in the congested airways. Alginate produced by the mucoid strains of *P. aeruginosa* poses an additional problem as it can act as a physical and ionic barrier preventing the antibiotic from reaching the bacteria. Most anti-pseudomonal antibiotics are administered intravenously but a major advance in therapy was the introduction of the first oral antibiotic, the quinolone, ciprofloxacin. Tobramycin is generally the aminoglycoside of choice and is frequently administered in a nebulised form, although it can be given intravenously. Recently, a large multicentred trial of highly pure nebulised tobramycin (TOBI) has shown particular success in reducing the normally progressive deterioration in lung function (Ramsay *et al*, 1999)

Ironically, 20% of isolates of *P. aeruginosa* from chronically colonised CF patients may exhibit antibiotic hypersusceptibility to β-lactams. These hypersusceptible *P. aeruginosa* are morphologically indistinguishable from resistant strains and both strains can be present in the same sputum (Irvin *et al*, 1981). Hypersusceptibility results from two chromosomal mutations known as *bls* and *tps* (Fyfe and Govan, 1984). An explanation for such bacteria is that hypersusceptibility represents a state of high permeability to cope with nutrient depletion with the bacterial biofilm.

7.4.7 Early diagnosis of *P. aeruginosa* in CF patients

It has been reported that antibodies specific to *P. aeruginosa* can be detected in serum prior to isolation of the organism from sputum, suggesting that only small numbers of organisms may be necessary for initial infection (Brett *et al*, 1988). Rapid detection of *P. aeruginosa* may be achieved by monoclonal antibody based immunoassay (Nelson *et al*, 1992) and by amplification of nucleic acid sequences specific to the *alg*D gene using PCR (McIntosh *et al*, 1992). These techniques can be applied directly to sputum or saliva and can detect small numbers of organisms which may not be detected by cultural methods. However, a disadvantage of the immunological and PCR-based methods is that there is no organism on which to carry out other tests such as antimicrobial sensitivities or epidemiological investigations. Another PCR technique using a species specific oligonucleotide probe derived from the variable regions of the nucleotide sequence of 16S rRNA from *P. aeruginosa* was developed (O'Callaghan *et al*, 1994). The probe itself is not particularly sensitive, however, sensitivity can be increased by amplification of the DNA using PCR followed by hybridisation of the PCR product with labelled oligonucleotide. Results showed full concordance between culture and the PCR and DNA hybridisation technique with a 100% sensitivity. A similar technique used by Karpati & Jonasson (1996) involved a primer based on the 16S rRNA sequences and confirmed the previous findings. *P. aeruginosa* was isolated by culture in three cases which were undetected by PCR; in contrast, PCR was positive in five cases in which the organism was not isolated by culture.

7.5 EPIDEMIOLOGY

Systems which allow reproducible and discriminating typing ('fingerprinting') of pathogens are essential tools in the epidemiology of microbial infection. Typing is particularly important in the case of ubiquitous opportunistic pathogens such as *P. aeruginosa* to determine whether infection is endogenous, exogenous and to investigate spread of an epidemic clone in an outbreak. In CF, bacterial typing has been used to determine whether individual strains of *P. aeruginosa* persist throughout the many years of colonisation despite characteristic phenotype changes in motility, mucoidy, LPS structure and serum susceptibility (Gilligan, 1991; Mahenthiralingam *et al*, 1996; Govan & Deretic, 1996). When a CF patient becomes reinfected with *P. aeruginosa*, following eradication due to antimicrobial therapy or transient colonisation, fingerprinting can establish whether the recent infection is due to the original strain or acquisition of a new strain.

The environmental sources from which CF patients acquire *P. aeruginosa* remain unclear. Environmental aquatic habitats have been shown to be a possible source of *P. aeruginosa* which subsequently colonise susceptible CF hosts (Romling *et al*, 1994; Leitao *et al*,1996). Although, Romling *et al* (1994) found similarities between strains of *P. aeruginosa* recovered from aquatic habitats, in several geographical areas, and those recovered from CF patients, the aquatic sources were at least 200Km from the CF centre. Tredgett *et al* (1990) found that the incidence of the most common bacteriocin (pyocin) types namely 1, 3, 5 and 10 in non-CF isolates, which accounted for 58-85% of strains of *P. aeruginosa*, differed significantly from CF

isolates which showed a higher incidence of rare types. These findings suggested the existence of a subpopulation of *P. aeruginosa* which have a predilection for pulmonary colonisation in CF. The properties of this subpopulation can only be speculated. However, it is interesting to note that the strain responsible for colonising two CF patients during a jacuzzi session was highly motile and mucinophilic (Govan & Nelson, 1992).

With the exception of siblings, CF patients are generally not thought to acquire *P. aeruginosa* by cross infection. However, evidence of small clusters of patients with the same strain has been reported from the Danish Centre (Hoiby & Rosendal, 1980). Combined results of serotyping and phage typing showed 13 clusters ranging from 2 - 10 patients within each cluster; each with distinct epidemiological types. One of the problems with this study was that many of these isolates shared polyagglutinability and rough LPS and thus would be expected to 'appear' similar by these phenotypic typing systems. However, further studies on some of the isolates were carried out using the genomic techniques of ribotyping and PFGE and confirmed the earlier findings and provided evidence of epidemic spread of *P. aeruginosa* amongst the Danish CF patients. In contrast, Grothues *et al* (1988) showed that, in general, only CF siblings were colonised with the same or closely related strains of *P. aeruginosa*; unrelated CF patients being typically colonised with different strains. In another study, it was shown that siblings are not always colonised with the same strain of *P. aeruginosa*. Renders *et al* (1997) studied six pairs of CF siblings over a 20-126 month period using arbitrarily primed PCR. They

found that two of the six pairs of siblings were each colonised with the same strains, which persisted over a ten year period. However, these authors also reported that the other pairs of siblings showed only transient cross-infection, probably from an environmental source in the home. In the mid 1980's, evidence of potential epidemic spread of an antibiotic-resistant strain of *P. aeruginosa* within CF patients was again observed in the Danish Centre (Pedersen *et al*, 1986). However, the study was based on phage and serotyping hence the hypothesis of a single strain being responsible for cross-infection was criticised. The incidence of *P. aeruginosa* cross infection among CF patients attending summer camps is minimal and the 'risk' considered to be trivial compared to the social and psychological benefits of these camps (Hoogkamp-Korstanje *et al,* 1995). There is no evidence of cross-infection due to *P. aeruginosa* in CF patients sharing hospital rooms (Speert & Campbell, 1987) or attending CF clinics on a regular basis (Govan & Nelson, 1992). Finally, in 1993 a working party comprising clinicians and scientists from Europe and North America reached the consensus that "Although cross-infection with *P. aeruginosa* does not seem to be a major problem in some hospitals, cross-colonization and epidemic spread of multidrug-resistant *P. aeruginosa* between CF patients has been observed in some studies" (Bingen *et al*, 1993).

7.5.1 Phenotypic typing systems for *P. aeruginosa*

Until recently, typing of *P. aeruginosa* for epidemiological purposes relied on phenotypic systems such as serotyping, phage susceptibility and bacteriocin production. Despite the advantages and fashionability of genomic fingerprinting

these three classical phenotypic systems are still widely used and can play an important role as screening techniques especially when epidemiological investigations involve large numbers of isolates.

7.5.2 Serotyping

Serotyping is based on the identification of the heat-stable O-specific LPS antigen of the outer membrane. Antisera to O antigens are obtained from rabbits and the O antigen type is determined either by slide agglutination or microtitre plate (Fisher *et al*, 1969; Pitt,1988). In 1983, an International Antigenic Typing System (IATS) was developed (Liu *et al*, 1983), establishing the existence of at least 17 major heat-stable somatic antigens and 3 new antigens have been recently added to the existing group (Liu & Wang, 1990). This typing system characterises most *P. aeruginosa* isolates possessing smooth LPS but shows poor discrimination against isolates with rough LPS which are common in CF patients. The majority of CF isolates, especially mucoid strains, are polyagglutinating or non-typeable due to the loss of O antigenic moiety of LPS (Hancock *et al*, 1983). Approximately 90% of isolates of *P. aeruginosa* from non-CF sources are typeable by serotyping and show fairly good discrimination.

7.5.3 Phage typing

Phage typing is based on lysis of bacteria by bacteriophage in which lytic patterns obtained by a set of phages against the 'test' isolate provide a lytic 'fingerprint' (Asheshov, 1974; Pitt, 1988). Approximately, 85% of clinical strains of

P. aeruginosa can be phage typed but the reproducibility is poor and phage typing is particularly unsuitable for CF isolates due to the loss of LPS receptors from the bacterial surface and the gelatinous growth of mucoid isolates.

7.5.4 Bacteriocin typing of *P. aeruginosa*

Bacteriocins are antibacterial proteins that are inactive against the producing strain but kill other strains of the same species and occasionally closely related species. The three categories of bacteriocin (pyocin) produced by *P. aeruginosa* are designated R, F and S. R-type pyocins resemble the tails of contractile phages and their bacterial receptor is LPS (Govan,1974a; Govan, 1974b). F-type pyocins are rod-shaped, flexuous and resemble the tail of non-contractile *Pseudomonas* phages. S-type pyocins are of low molecular weight and are generally trypsin sensitive although a trypsin resistant category resembling the enterobacterial microcins has also been described (Darrell & Wahba, 1964; Govan, 1978). The nature of receptor for both F and S-type pyocins remains unknown although the F receptor is thought to lie within LPS. S-type pyocins diffuse readily through agar resulting in the production of a wide zone of inhibition on solid media in contrast to R- and F-types which produce a narrow zone of growth inhibition around the producer strain (Govan, 1978). R and F pyocins are produced by over 90% of clinical isolates with the S-type found in about 70% of strains (Govan, 1986). The inhibition patterns produced by pyocins from a 'test' isolate against the eight indicator strains form the basis of the 'cross-streak' pyocin typing technique described by Gillies and Govan (1966). This typing method identifies 105 pyocin types which can be further

subdivided by the use of 5 subtype indicator strains (Govan, 1978). The original method was later revised by Fyfe *et al* (1984) who introduced an overlay technique resulting in a more rapid procedure. Pyocin typing is readily applicable to isolates of *P. aeruginosa* including those from CF patients; approximately 90% of all clinical and environmental isolates are typeable by this method.

7.5.5 Genomic typing of *P. aeruginosa*

Genomic typing systems applicable to *P. aeruginosa* include use of gene probes, restriction fragment length polymorphism (RFLP) and PCR. Use of a DNA probe based on the exotoxin A (ETA) gene was probably one of the first genomic typing systems applied to *P. aeruginosa*. Most *P. aeruginosa* possess the ETA structural gene which consists of a conserved region which is common to most strains of *P. aeruginosa* and a variable region. The typing probe is a 741 base pair *Pst*I-*Nru*I fragment from the variable region upstream of ETA. As a typing tool, the probe has been shown to be more discriminatory than serotyping, biotyping, antibiograms and colonial morphology; isolates indistinguishable by these systems were subdivided using the probe and isolates found to have different antibiograms were shown to be the same using the probe (Olgle *et al*, 1987). Interestingly, pyocin typing and the ETA gene probe were used together to produce the first documented evidence for clonal transition from the non-mucoid to the mucoid form within three months (Govan, 1990).

7.5.6 Random arbitrarily primed DNA (RAPD) PCR

Random arbitrarily primed DNA PCR has been applied to numerous epidemiological investigations of *P. aeruginosa*. In the majority of cases this fingerprinting system has been compared to PFGE with RAPD demonstrating equal discrimination (Bingen *et al*, 1996; Mahenthiralingam *et al*, 1996). In contrast, RAPD was shown to be less discriminating than PFGE when examining closely related isolates of *P. aeruginosa* from CF patients (Kersulyte *et al*, 1995). When RAPD, serotyping, phage and pyocin typing were used to investigate the incidence of cross infection of *P. aeruginosa* among CF patients attending summer camp, RAPD was found to be more discriminating than any of the other typing techniques investigated (Hoogkamp-Korstanje *et al*, 1995). The primers used for RAPD are important as some primers show reduced resolution compared to PFGE. An advantage of RAPD over PFGE is that the template DNA need not be high molecular weight, double stranded or high purity and only nanogram amounts are required.

7.5.7 Flagellin gene polymorphisms

The flagellin gene is a widely applicable and useful genetic marker for studying genetic variation within populations of closely related bacteria. Two distinct flagellin protein antigenic types, a and b, are found in *P. aeruginosa* (Allison *et al*, 1985). Flagellin gene sequence analysis has shown that variation is most apparent in the central region of the flagellin protein. Oligonucleotide primers were developed, specific to the N and C-terminals of this conserved region, and used to amplify by PCR the central region of the flagellin protein (Winstanley *et al*, 1996). The

resultant PCR product was digested using seven different restriction enzymes prior to electrophoresis. Isolates of *P. aeruginosa* were differentiated according to the RFLP pattern produced. This technique requires relatively simple equipment, is reproducible and rapid to perform as results are available within five to six hours. However, flagellin gene polymorphisms have been reported to be less discriminating than other DNA-based methods (Grundmann *et al*, 1994).

7.5.8 Field-inversion gel electrophoresis (FIGE)

The technique field-inversion gel electrophoresis has been applied in a number of epidemiological studies of *P. aeruginosa* (Grothues and Tummler, 1987; Tummler *et al*, 1991). Grothues *et al* (1988) found FIGE to be more discriminating than serotyping, phage and pyocin typing. The number of DNA fragments observed after electrophoresis was dependant on the restriction enzyme used with *Spe*I producing the fewest bands. *Spe*I digestion makes interpretation easier; however, the cost of this enzyme limits its use. This study also stated that strains were classified as being clonally related even with up to a six band difference. Ojeniyi *et al* (1991) used a number of typing systems, including FIGE, to fingerprint polyagglutinating strains of *P. aeruginosa* from CF patients and found that the discriminating power of FIGE was even higher than that for the ETA probe and both systems showed poor correlation with all the phenotypic techniques used.

7.5.9 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis has been shown to be the most discriminating method of genomic typing for *P. aeruginosa*. Grundmann *et al* (1995) confirmed that PFGE showed the highest discriminatory power than either the exotoxin A probe or ribotyping. Grundmann *et al* also suggested that up to a four band difference in banding patterns, using restriction enzyme *Spe*I, indicates that isolates are related.

7.5.10 Ribotyping

Ribotyping has been used in a number of epidemiological studies of *P. aeruginosa.* The discriminatory ability of this technique is dependant on the restriction enzyme used with *Pvu*II showing the highest discrimination and *Bam*HI and *Eco*RI having the lowest (Gruner *et al*, 1993; Bennekov *et al*, 1996; Nociari *et al*, 1996). The profiles produced by ribotyping are composed of strong and weak bands. The weak bands produced by *Pvu*II, *Hind*III and *BamH*I are reproducible in contrast to those produced by *EcoR*I. Use of additional genomic typing systems suggests that a single band difference within a ribotyping profile indicates that an isolate is a different genotype. Bingen *et al* (1992) used ribotyping and esterase electrophoresis (ETT) to investigate the relatedness of CF isolates before and after antimicrobial therapy and observed that a CF patient remained colonised with a single strain of *P. aeruginosa* even after several courses of antibiotics. Both systems showed a good degree of discrimination but ETT was sightly more discriminating than ribotyping. Although several studies have stated that ribotyping is highly discrminatory, Bingen *et al* (1996) demonstrated that multiresistant strains of *P. aeruginosa* serotype O:12 were

indistinguishable by ribotyping, in comparison to the discriminatory ability of both RAPD and PFGE.

A fully automated ribotyping system has recently been developed, RiboPrinter (TM) Microbial Characterization System (DuPont) and allows the typing of 32 isolates in 8 hours (Pfaller *et al*, 1996). In comparison with PFGE, this system is less sensitive in discriminating different strains of *P. aeruginosa* than when used for typing *Escherichia coli*.

A number of other typing systems have been used to fingerprint *P. aeruginosa*; these include plasmid profiles, multilocus enzyme electrophoresis and pyrolysis mass spectrometry.

7.5.11 Comparison of typing systems for *P. aeruginosa*

In the early 1990's, a multicentre study examined 11 techniques then available for typing strains of *P. aeruginosa*. Typeability, reproducibility and discriminatory power was determined for each of the methods. The typing systems included several serotyping methods, phage susceptibility, bacteriocin production, pilin gene typing, and analysis by RFLP using exotoxin A probe. The study involved typing 200 strains comprising 150 CF isolates and 50 isolates from non-CF infections, vegetables and river water. RFLP analysis with the exotoxin A probe was determined to be the method of choice for strains from CF patients having rough LPS although LPS-based serotyping methods were effective for typing non-CF strains.

RFLP analysis also showed the highest degree of discrimination, good reproducibility and was the only technique able to type all 200 strains (Speert *et al*, 1994). It must be noted that PFGE was not included in this comparison of typing systems. A key feature of this study was that each of the 200 isolates was examined 'blindly' on three occcasions.

7.6 AIMS

The overall aim of this section of the thesis is to apply PFGE to investigate, and hopefully resolve, two important features of *P. aeruginosa* epidemiology.

1. Although mucoid *P. aeruginosa* have long been considered to be variants arising from colonisation by classic non-mucoid *P. aeruginosa*, there is surprisingly little documented evidence of a clonal relationship based on the use of reliable genomic typing techniques. The first aim of this section of the thesis was to apply, develop and test the use of PFGE to investigate isolates of *P. aeruginosa* cultured during the initial stages of colonisation and continuing longitudinally to the emergence and chronic colonisation by mucoid *P. aeruginosa*.

2. In contrast to *B. cepacia* epidemiology, there has been little scientific evidence that cross-infection plays a significant role in the prevalence of *P. aeruginosa* amongst CF patients, with the important exception of CF siblings. During the early stages of this thesis, an apparent outbreak of cross-infection in Liverpool involving a β-lactam resistant *P. aeruginosa* provided an important opportunity for investigation. The second aim of this section was to use PFGE to investigate the epidemiology of

the Liverpool isolates in conjunction with a Liverpool-based typing study based on flagellin gene polmorphisms.

7.7 RESULTS

7.7.1 The application of PFGE to isolates of *P. aeruginosa*

Prior to the application of PFGE for epidemiological studies of *P. aeruginosa* the discriminatory power of the technique and various procedural parameters were investigated using a collection of clinical and environmental isolates. Initially, three different restriction enzymes, *Xba*I, *Dra*I and *Ssp*I (AATATT), were used to digest chromosomal DNA prior to the separation of DNA fragments by PFGE. A portion of the same plug, from an isolate of *P. aeruginosa*, was digested by each restriction enzyme. All three enzymes produced 25 or more DNA fragments, ranging from < 48.5kb to 436.5kb. Restriction enzyme *Ssp*I produced numerous low molecular weight bands which were close together on the gel and were difficult to distinguish. Although *Dra*I produced the smallest number of DNA fragments, the bands appeared considerably fainter than those produced by *Xba*I. After considerable 'preliminary' studies, *Xba*I was selected as the enzyme of choice for genotyping isolates of *P. aeruginosa* in this thesis based on the quality of the PFGE profiles. Fig. 7.1 illustrates representative PFGE profiles and demonstrates the discriminatory power of this technique for isolates of *P. aeruginosa*.

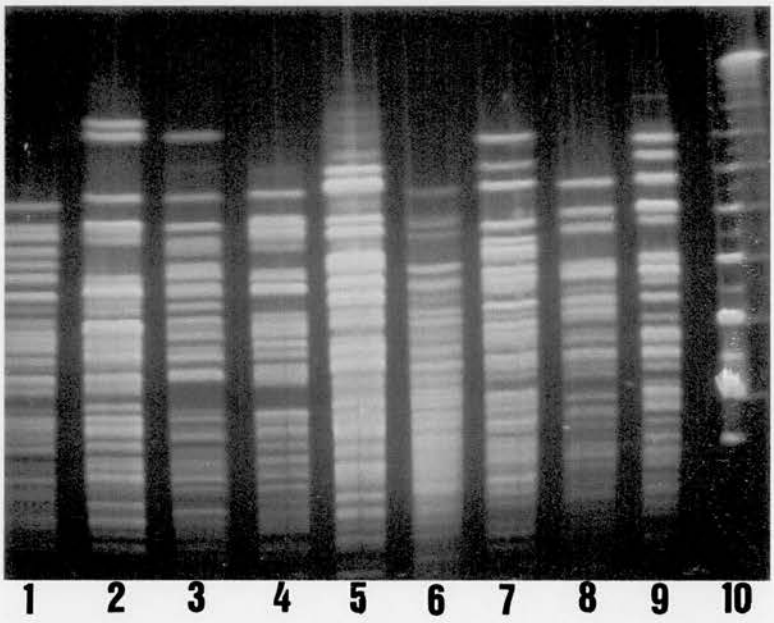


FIG. 7.1 *Xba*I digestion of DNA from nine epidemiologically unrelated isolates of *P. aeruginosa*, from CF patients. Lanes 1 to 9, H133, C1001, C2270, C1224, C763, C1334, C518, C1864, C907; lane 10, lambda concatemers (size range 48.5kb to 582 kb, in increments of 48.5kb)

7.7.2 Investigation of isolates of *P. aeruginosa* cultured from CF patients during chronic infection.

This investigation involved the use of pyocin typing and PFGE to type the initial or early isolate of *P. aeruginosa* and isolates obtained from the same patient at least two years and up to 11 years later. Isolates of *P. aeruginosa* were obtained from 44 CF patients, comprising 18 paediatric and 26 adult patients. Typing results are summarised in Table 7.1.

In only one case did the paired isolates of *P. aeruginosa* fail to produce a pyocin type. According to the pyocin typing results, only 6 (14%) of the 43 CF patients produced identical pyocin types in both isolates of *P. aeruginosa* cultured from the same patient over a period of time. However, further comparison of the pyocin types, produced by paired isolates, revealed that in four cases the only difference was variation in the activity of s-pyocins which are of low molecular weight and produce large zones of inhibitory activity. The evidence from this study suggests that production of s-type pyocins is unstable as most paired isolates failed to show the same pattern of 's' activity. If 's' activity is ignored a further four paired isolates have the same pyocin type.

TABLE 7.1. Bacteriocin typing and PFGE results from paired isolates of *P. aeruginosa* from 44 CF patients.

Patient	Bacteriocin type		Time interval (yrs)	PFGE
	1st isolate	2nd isolate		
AA	10/b (s-3,7)	10/c (s-6,d)	3	same
FB	u/c/a (s-2,a,b)	9/u/c (s-a,b)	3	different
VD	9/u/c	9/j	8	ut
SD	9/j (s-5)	9/u/c (s-7)	10	ut
VE	3/g (s-5)	3/g (s-5)	2	same
EG	3/g (s-2)	35/c (s-7)	3	different
CG	3/c	3/g	5	same
MG	3/g	5/u/c	3	different
SG	5/f (s-5)	44/f	4	different
RG	19/u (s-7)	u/c/h (s-7,b)	6	same
JH	9/f (s-7)	9/f (s-5)	7	same
CJ	29/x	3/a (s-d)	2	different
LL	3/s	3/c	4	same
CL	3/a	3/a (s-a,b,d)	4	same
SL	3/a	3/n	4	same
RM	1/c	1/c	3	same
FM	35/e	45/e (s-b)	9	same
JW	105/h (s-3,7)	5/u/c	3	different
DB	33/c (s-3,7)	101/c	7	different
TB	92/r (s-5)	5/v	2	same
LB	31/v (s-7)	ut	9	different
FC	u/c/v	ut	4	same
SD	10/a (s-7)	10/a (s-5,a)	5	same
MD	10/d	10/d	9	same
CF	5/u (s-5)	5/u (s-5)	5	same
BF	1/d (s-7)	1/w (s-b)	4	different
GI	60/v	60/f (s-7)	6	same
SJ	u/c/f	6/v (s-5)	11	different
AK	31/v (s-7)	29/e	11	same
DK	u/c	ut/v	2	same
JMcE	6/v (s-3,4,5)	5/x	7	different
JM	10/a (s-5,a,b,e)	u/c/r (s-7)	11	different
BM	3/n	3/n	4	same
NM	10/h	3/b	8	different
JMn	3/e	3/n	10	same
DN	ut	ut	6	same
DO'N	1/a (s-b)	10/a	4	different
CO	10/a (s-3,7)	u/c/x (s-7)	10	different
VS	6/c	1/x	4	same
PS	49/k	50/k	8	same
AS	10/c (s-7,e)	10/c	6	same
RT	1/p	45/b (s-5)	8	different
AW	3/a	3/a	4	same
FW	50/v	u/c/v	4	same

u/c unclassifiable type; ut untypable

Pyocin typing results can sometimes prove difficult to read especially when strains produce faint zones of inhibition. When two isolates only differ by the inhibition pattern of a single indicator they are generally classified as the same strain. Consequently, a further 8 patients would be regarded as having the same strain of *P. aeruginosa* in the two isolates. In conclusion, 18 (42%) of the 43 CF patients could be regarded as harbouring the same strain of *P. aeruginosa* after at least two years colonisation.

Surprisingly, two isolates were untypeable by PFGE, producing only a smear in the lane of the agarose gel. The same result was obtained on repeated investigation involving digestion with other restriction enzymes using a different procedure for DNA extraction. Failure to obtain a 'PFGE result' is unusual as very few isolates are untypeable by PFGE. Interestingly, both isolates were recovered from CF siblings and although they failed to produce a PFGE profile the isolates produced pyocin type patterns differing by a single sub-type indicator and 's' activity, suggesting that they are the same strain of *P. aeruginosa*.

Comparison of the PFGE profiles produced by the paired isolates from the other 42 CF patients revealed that 26 (59%) of the patients harboured the same strain of *P. aeruginosa* for considerable periods of time, in some cases for 11 years. Isolates of *P. aeruginosa* from the remaining 16 patients were found to be different by PFGE, suggesting that re-infection with a different strain had occured.

When the data from pyocin typing and PFGE were compared; paired isolates of *P. aeruginosa* from 26 patients were shown to have the same strain by PFGE whereas only 18 patients were found to have the same strain by pyocin typing. However, the paired isolates of *P. aeruginosa* from the 16 patients which were shown to have different PFGE profiles also produced different pyocin types with the exception of one patient, D O'N. The pyocin types of both isolates from D O'N only differed by inhibition of a single indicator strain suggesting that both isolates belong to the same strain.

7.7.3 Are mucoid isolates a variant of the non-mucoid form?

Despite a surprisingly lack of scientific evidence, it has long been assumed that mucoid colonial morphotypes of *P. aeruginosa* isolated from CF patients are derived from the original classic non-mucoid form, responsible for initial colonisation. In this study, the initial non-mucoid isolate of *P. aeruginosa*, recovered from sputum culture, was compared to the first isolate of the mucoid phenotype in 12 CF patients. Results of fingerprinting by PFGE showed that in six patients the mucoid and non-mucoid forms produced identical PFGE profiles suggesting that they were the same strain of *P. aeruginosa*. However, isolates from the remaining six patients produced different PFGE profiles. In the interpretation of these results it is important to bear in mind that the time for conversion from the non-mucoid to the mucoid form varied from patient-to-patient with a range varying from 3 months to 10 years (Table 7.2).

TABLE 7.2. Time taken for conversion from the non-mucoid to the mucoid phenotype in isolates of *P. aeruginosa* and PFGE relationship between the isolates.

Patient	Conversion time	PFGE profile
FC	3 mths	same
WC	6 mths	same
TG	7 mths	different
JH	9 mths	same
CJ	2 yrs	same
PO	2 yrs	different
AG	3 yrs	different
DB	4 yrs	same
GI	4 yrs	same
JM	4 yrs	different
NM	8 yrs	different
CO	10 yrs	different

In general, the shorter the period of time for conversion from the non-mucoid to the mucoid phenotype the more likely the strains were to be the same. However, in one CF patient, only seven months elapsed prior to the emergence of the mucoid form of *P. aeruginosa* and these isolates were found to be clonally unrelated by PFGE. In two other patients, both phenotypes were found to be identical despite a time interval of four years between first culture of non-mucoid *P. aeruginosa* and conversion to the mucoid form. As might be expected, in the two CF patients where conversion occured at eight and 10 years the non-mucoid and mucoid phenotypes were found to be different.

A further four CF patients were investigated where non-mucoid and mucoid forms of *P. aeruginosa* were isolated from sputum culture simultaneously; in all cases, both colonial morphotypes produced identical PFGE profiles. An interesting case involved a non-mucoid isolate of *P. aeruginosa* (C1385) which was cultured from a

CF patient who apparently became colonised after using a jacuzzi which contained the colonising strain. Within three months the patient was found to harbour a mucoid form (C1532) which when typed by PFGE showed only a two-band difference from the original non-mucoid isolate (Fig. 7.2).

To test the reliability of PFGE further, it was decided to make use of the large collection of isogenic derivatives of the *P. aeruginosa* genetic type strain PAO1 held in the strain repository of the Edinburgh CF Microbiology Laboratory. The isolates investigated (Table 7.3) comprised the wild-type parent strain PAO1 and a selection of variants chosen to include auxotrophy, antibiotic resistance, the presence of the conjugative plasmid FP2 and a series of *muc* mutations known to be responsible for the mucoid phenotype. These include mucoid mutants of *P. aeruginosa* isolated *in vitro* and mucoid isolates from CF patients (Fyfe and Govan, 1980; Deretic *et al*, 1993; Govan and Deretic, 1996; Boucher *et al*,1997). PAO 568, PAO 578, PAO 579 and PAO 581 represent the major independent *muc* loci which act as master switches for alginate regulation; they also cover the range of nutritional and environmentally responsive phenotypes observed in mucoid *P. aeruginosa* cultured from CF patients (Govan *et al*,1983; Govan and Deretic, 1996). PAO 552 and PAO 553 represent two classes of mutation found in non-mucoid revertants following loss of the mucoid phenotype. In view of the aims of this thesis, it was interesting (and reassuring) to observe that despite the multiple genetic and phenotypic differences in these PAO isolates, all of the strains produced the same PFGE profile (Fig. 7.3)

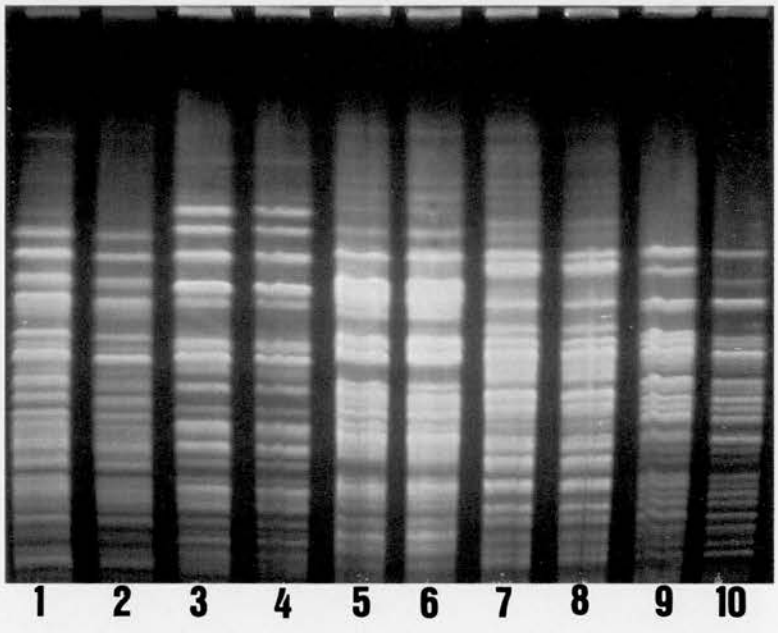


FIG. 7.2. *Xba*I digestion of DNA from isolates of non-mucoid (NM) and mucoid (Muc) *P. aeruginosa* from 5 different CF patients. Lanes 1 to 10, patient A (C1367 NM, C1387 Muc), patient B (C1158 NM, C1390 Muc), patient C (C748 NM, C904 Muc), patient D (C742 NM, C1377 Muc), patient E (J1385 NM, J1532 Muc).

TABLE 7.3. Type strain PAO1 and a selection of derivatives

Strain	Genotype / Description	Reference
PAO1	Prototype, *chl*-Z	Holloway (1969)
PAO 381	*leu*-38, *strep*-2, FP2	Stanisich & Holloway (1969)
PAO 568	*leu*-38, *strep*-2, FP2, *muc*-2	Fyfe & Govan (1980) Deretic *et al* (1993)
PAO 578	*leu*-38, *strep*-2, FP2, *muc*-22	Fyfe & Govan (1980)
PAO 579	*leu*-38, *strep*-2, FP2, *muc*-23	Fyfe & Govan (1980)
PAO 581	*leu*-38, *strep*-2, FP2, *muc*-25	Fyfe & Govan (1980)
PAO 552	Non-mucoid revertant of PAO 579 retaining original *muc*-23	Made by J.R.W. Govan
PAO 553	Non-mucoid revertant of PAO 579 caused by *muc*-23 repair	Made by J.R.W. Govan

leu, leucine requirement; *chl*, resistance to chloramphenicol; *strep*, resistance to streptomycin; FP2, contasins conjugative plasmid FP2; *muc*, mutation in alginate regulation.

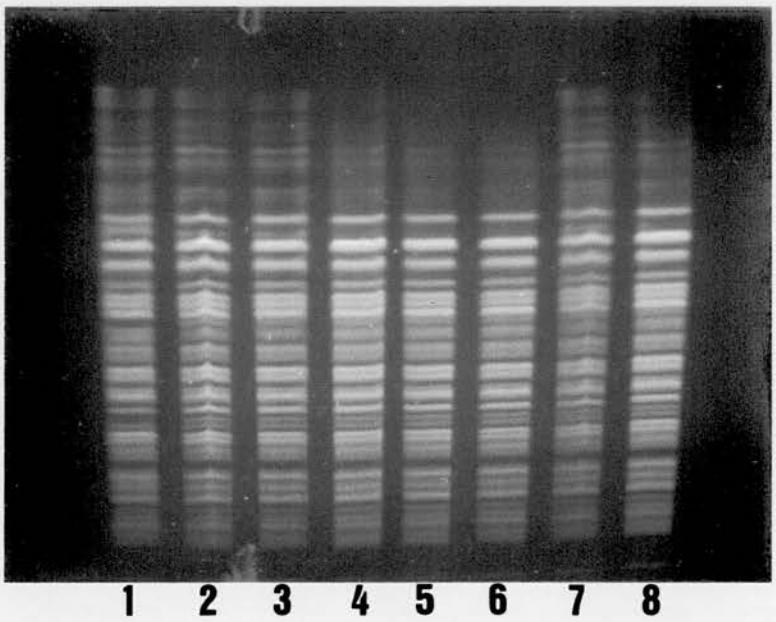


FIG. 7.3. *Xba*I digestion of DNA from *P. aeruginosa* PAO1 and its derivatives. Lanes 1 to 8, PAO1, PAO568, PAO578, PAO581, PAO579, PAO552, PAO553, PAO381.

7.7.4 Evidence of transmission of *P. aeruginosa* among CF patients.

As part of a multicentred antibiotic trial of cefpirome, a large number of isolates of *P. aeruginosa* were received from Liverpool CF patients. In total, 246 isolates were obtained from 72 CF patients. Sensitivity testing of these isolates showed that a large proportion were resistant to cefpirome and to other β-lactams, including ceftazidime. To investigate the relatedness of the Liverpool isolates, pyocin typing was used as a primary screen. A large proportion of the β-lactam isolates produced a pyocin type 45/e pattern and this clonal relationship was confirmed by PFGE. Isolates which produced pyocin types other than type 45/e were regarded as being different strains but any isolate which produced doubtful results or pyocin types similar to 45/e was examined by PFGE.

PFGE showed that isolates of *P. aeruginosa* from 53 of the Liverpool patients produced identical PFGE profiles. This unexpected result suggested that they were the same strain and providing the first compelling scientific evidence for cross-infection of *P. aeruginosa* in CF patients based on DNA fingerprinting. Isolates from a further eight patients produced up to a three band difference in their PFGE profiles and based on the criteria suggested by Tenover *et al* (1995), were regarded as being clonally related. The overall typing data indicated that 61 of the 72 Liverpool CF patients were colonised with the same strain of *P. aeruginosa.* Figure 7.4 shows the PFGE profiles of six isolates of *P. aeruginosa* recovered from CF patients in Liverpool including similar profiles produced by three β-lactam resistant isolates and three other profiles from Liverpool patients not harbouring the 'epidemic' strain. FIG.

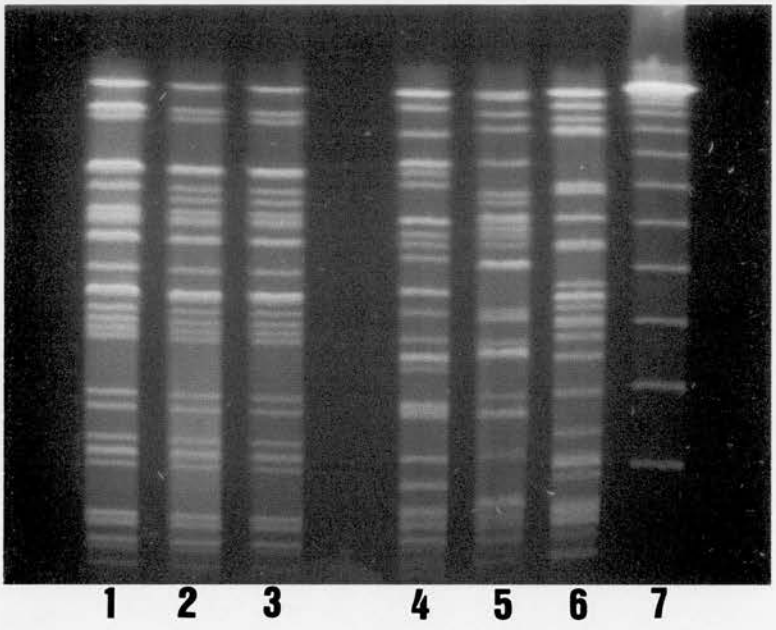


FIG. 7.4. *Spe*I digestion of DNA from six isolates of *P. aeruginosa* from different CF patients in Liverpool. Lanes 1 to 3, transmissible strain; lanes 4 to 6, different strains of *P. aeruginosa*; lane 7, lambda concatemers (size range 48.5kb to 582kb, in increments of 48.5kb).

Since this outbreak was to have important implications for the future management of *P. aeruginosa* infection in CF patients it was reassuring to note that a parallel investigation by the Liverpool team using flagellin gene polymorphisms produced concordant results (Cheng *et al*, 1996).

Rather disturbingly, following the Liverpool outbreak isolates of *P. aeruginosa* showing identical or similar PFGE profiles to that of the Liverpool strain have been observed in CF patients attending CF centres in Manchester, Nottingham and Edinburgh. There is also epidemiological evidence of transmission of this strain between two patients in Manchester, one of whom had previously attended the CF clinic in Liverpool (personal communication, Dr T. Stanbridge). Interestingly, there is also evidence that the Edinburgh patient has harboured this *P. aeruginosa* in her sputum for eight years with no evidence of transmission to any other CF patient. A distinguishing feature of this strain is its ability to grow on the commercially available *B. cepacia* medium.

7.8 DISCUSSION

In this study, investigation of *P. aeruginosa* cultured longitudinally from the same CF patient, and extending from initial through to chronic colonisation, showed some discrepancies between the results of pyocin typing and PFGE. Results of pyocin typing could be interpreted to suggest that this technique can distinguish between paired isolates which were found to be clonally-related by PFGE. However, a more likely explanation is that the differences in pyocin typing patterns between the paired isolates reflect lack of reproducibility of this simple phenotypic technique either in the sensitivity of the indicator strains or loss of pyocin production; this particular instability applies to s-type pyocins. Phenotypic changes in *P. aeruginosa* during chronic colonisation or by storage at - 70^{o}C has been shown to be responsible for loss of pyocin production (Romling *et al*, 1994). In this study, the initial isolates of *P. aeruginosa* were stored at -70^{o}C prior to fingerprinting whereas the later isolates were generally typed following sub-culture of fresh sputum isolates.

Examination of paired isolates of *P. aeruginosa* from the same CF patient indicates that the majority of patients (59%) harboured the same strain for extended periods. However, in some patients the original strain appeared to have been replaced. Other investigators, have reported that most CF patients are colonised with a predominant strain of *P. aeruginosa*, although some patients may harbour more than one strain (Romling *et al*, 1994; Godard *et al*, 1993; Loutit & Tompkins, 1991). However, most of these studies involved small numbers of CF patients. Similar results to the ones observed in this study were found by Speert and collegues (1989) in a study of

23 CF patients over a seven year period. Thirteen of the patients were colonised with the same strain of *P. aeruginosa* while the remaining 10 patients were colonised with at least two different strains of *P. aeruginosa*.

A possible explanation for the results obtained in this thesis, and those reported by Speert *et al* (1989) could be the presence of mixed infection with different *P. aeruginosa* strains being fingerprinted on each occasion. Alternatively, it could be argued that it would be realistic to expect differences in paired isolates when the time interval was extensive, for example in some instances, extending to ten-years.

In each of the two sets of siblings SD, VD and FM, JMn the strain of *P. aeruginosa* was found to be the same either by pyocin typing in the case of SD and VD or by PFGE in siblings FM and JMn. The isolates recovered from SD and VD were untypeable by PFGE, which is probably due to the high content of endogenous nuclease present, indicated by the presence of a smear in the lane of the PFGE gel. Although it is unusual for microorganisms to be untypeable by genomic methods, similar findings have been reported (Romling *et al*, 1994) and have also been observed in a small number of isolates of *B. cepacia* (personal observation).

Genomic fingerprinting by PFGE also suggested that non-mucoid and mucoid isolates of *P. aeruginosa*, from the same CF patient were not always clonally-related. In the four patients, where mucoid and non-mucoid variants were present on the first *P. aeruginosa* culture the PFGE profiles were identical. This provides additional

evidence to confirm the long-standing speculation that the mucoid phenotype is a variant of the non-mucoid form. Similar findings have been observed when mucoid and non-mucoid isolates of *P. aeruginosa*, from the same specimen, have been fingerprinted by serotyping and pyocin typing (Bergen & Hoiby, 1975) and by PFGE and FIGE (Romling *et al*, 1994).

The fact that fingerprinting by pyocin typing and PFGE showed non-mucoid and mucoid isolates to be different in half the patients could be explained by subsequent colonisation by a second *P. aeruginosa* prior to conversion to the mucoid phenotype. The time interval prior to conversion from non-mucoid to the mucoid phenotype is highly variable. In patient TG, only seven months elapsed between the appearance of the first non-mucoid isolate of *P. aeruginosa* and the emergence of the mucoid variant. Conversely, two patients DB and GI were colonised with non-mucoid *P. aeruginosa* for four years prior to the emergence of the mucoid phenoytpe and both colonial morphotypes demonstrated identical PFGE profiles.

In contrast to the widely accepted transmissibility of *B. cepacia* amongst CF patients, the issue of transmission of *P. aeruginosa* has until recently remained highly controversial. Several prospective studies have found no evidence of cross-infection either in CF centres or during summer camps. Furthermore, in 1993, a consensus report drawn up following a workshop on the epidemiology of CF pulmonary infection contained the statements: "At present, there are more studies suggesting person-to-person transmission of *B. cepacia* than of *P. aeruginosa*" and "cross-

infection with *P. aeruginosa* does not seem to be a problem" (Bingen *et al*, 1993). Thus with the important exception of CF siblings it might be reasonable to conclude that *P. aeruginosa* cross-infection does not present a major problem in the management of CF patients (Grothues *et al*, 1988). However, generalisations are always dangerous in CF microbiology and small clusters of patients apparently harbouring the same strain of *P. aeruginosa* have been reported in Denmark (Hoiby & Rosendal, 1980; Pedersen *et al*, 1986). It must be noted, however, that these findings were based on the phenotypic systems of serotyping and bacteriophage typing, which as previously discussed are particularly inappropriate for typing CF isolates of *P. aeruginosa*.

The evidence for *P. aeruginosa* cross-infection in CF patients in Liverpool is compelling both in the large number of patients involved, and in the concordance observed with the two genomic but distinct typing systems, namely PFGE and flagellin gene polymorphisms. Unfortunately, the identification of the origins and modes of spread of this epidemic strain were hindered by the retrospective nature of the investigation and remain unclear. Interestingly, many patients had previously received monotherapy with ceftazidime which was thought to be responsible for the emergence of β-lactam resistance. If true, this explanation demonstrates the importance of the recommendation of the use of at least two antibiotics for therapy to minimise the risk of emergence of resistant strains (Livermore, 1989; Pitt, 1986). However, the mere development of resistance following monotherapy does not explain the spread of a single strain of *P. aeruginosa* amongst the Liverpool patients.

Had monotherapy been the major reason for β-lactam resistance, then the typing results should have indicated a large number of individual strains within the Liverpool isolates. An intriguing and worrying posibility is that the 'Liverpool' strain resembles an unusual highly transmissible lineage of *P. aeruginosa* resembling the highly transmissible and notorious lineage of *B. cepacia* referred to as ET12. Further studies on the 'Liverpool' *P. aeruginosa* might prove fruitful. One possibility is that this interesting and clinically-relevant *P. aeruginosa* be put forward as a candidate for the sequencing of a second *P. aeruginosa* following the recently complete sequencing of PAO1.

CHAPTER 8

BURKHOLDERIA CEPACIA

8.1 TAXONOMY AND NOMENCLATURE

Burkholderia cepacia (synonyms *Pseudomonas cepacia, Pseudomonas multivorans, Pseudomonas kingii,* and eugonic-oxidizer group I) was first recognised as a phytopathogen, causing soft rot of onion bulbs (Burkholder, 1950). In 1960, a taxonomic study of aerobic pseudomonads revealed a distinctive cluster of isolates within the Pseudomonas rRNA homology group III which were capable of utilising a wide range of organic compounds. The name *P. multivorans* was designated to this group of isolates (Stanier *et al*, 1966). Subsequently, it was realised that similar isolates, causing soft rot in onions, had already been described and the designation *P. cepacia* (Latin: *cepa* = onion) was given precedence (Palleroni & Holmes, 1981).

On the basis of cellular lipid and fatty acid composition, 16S rRNA sequences, DNA-DNA homology values and phenotypic characteristics,Yabuuchi *et al* (1992) proposed that *P. cepacia* and six other members of the rRNA homology group II (*Pseudomonas mallei*, *Pseudomonas pseudomallei*, *Pseudomonas caryophylli*, *Pseudomonas gladioli*, *Pseudomonas pickettii*, *Pseudomonas solanacearum*) be transferred to the new genus *Burkholderia* with *B. cepacia* as the type-species. This proposal was subsequently validated in 1993 (List No. 45). Two species, *B. pickettii*

and *B. solanacearum* have since been transferred to the genus *Ralstonia* (Yabuuchi *et al*, 1995). Further possible changes to the genus *Burkholderia* are currently under investigation.

B. cepacia isolates presumptively identified by conventional techniques have recently been further divided by polyphasic taxonomy, comprising DNA-DNA hybridisation and whole cell protein analysis, into five genotypic subgroups refered to as genomovars I -V (Vandamme, 1995). All five genomovars form a group of closely related organisms known as the *B. cepacia* complex. The term genomovar was introduced to denote phenotypically similar but genotypically different groups of bacteria; according to taxonomic conventions, new species names cannot be given to bacteria that cannot be identified by phenotypic characteristics (Ursing *et al*, 1995). Genomovar V has been designated *B. vietnamiensis*, based on its nitrogen-fixing properties.

There is also sufficient biochemical information to propose a species name for genomovar II isolates; namely *B. multivorans* (sp. nov. mul. ti' io.rans L. ajd. *multus*, much; L. part. ajd. *vorans*, devouring, digesting; M. L. part ajd. *multivorans*, digesting many compounds). *B. multivorans* has been cultured from sputum samples from CF patients but can also cause infection in non-CF patients; the organism is also present in the hospital environment and in soil (Vandamme *et al*, 1997).

Genomovar III has been divided into two subgroups or biovars with a third biovar consisting of non-saccharolytic strains. Although isolates from CF patients can be found in each of the genomovar groups, a high proportion belong to genomovar III and this includes most strains associated with epidemics and those responsible for 'cepacia syndrome'. Until recently, no environmental isolates of *B. cepacia* had been found in genomovar III. However, a striking piece of new taxonomic evidence indicates that a major group of soil isolates thought to represent a new species of *Burkholderia* are in fact in the genomovar III group within the *B. cepacia* complex. This observation provides the first compelling scientific evidence of the close relationship between soil isolates of the *Burkholderia* complex and genomovar III, arguably the most virulent and transmissible members of the complex (personal communication, P. Vandamme).

8.2 GENERAL CHARACTERISTICS

For the purpose of this thesis, and unless stated otherwise, the term *B. cepacia* will be used to represent all five members of the *B. cepacia* complex including *B. multivorans* and *B. vietnamiensis*. *B. cepacia* is non-sporing, strict aerobic Gram-negative bacillus and is motile with a tuft of polar flagella. Most strains are catalase and oxidase positive and produce various non-fluorescent, diffusible pigments. Pigmentation ranges from greyish white or yellow to reddish purple or brown and is often dependent on the carbon source. The transcontinental 'epidemic' strain of *B. cepacia* is sometimes refered to as the ET12 lineage, and belongs to genomovar III, produces a characteristic dry, rough colonial appearance and a dark-brown

melanin-like pigment; this pigment is rarely found in other strains of *B. cepacia*. Production of this pigment is enhanced with the use of a tyrosine-containing medium, including Furunculosis agar (Ogunnariwo & Hamilton-Miller, 1975). The optimum temperature for growth of *B. cepacia* is 30^{o} - 35^{o}C although some strains can grow at 42^{o}C; none grow at 4^{o}C although they can survive at this temperature. Characteristically, *B. cepacia* only survive for 3 - 5 days on nutrient agar although they survive longer on minimal media or on charcoal slopes or plates.

In general, *B. cepacia* are relatively uniform in the following biochemical characteristics; gelatin liquifaction, hydrolysis of o-nitrophenyl β-D-galactopyranoside and aesculin, nitrate reduction to nitrite, lysine decarboxylase, production of poly-β-hydroxybutyrate and arginine dihydrolase is not produced. Additional biochemical characteristics, frequently present in isolates of *B. cepacia* from CF patients, include production of ornithine decarboxylase, C14 lipase, alginase, trypsin, hydrolysis of urea and xanthine (Govan & Deretic, 1996). Interestingly, the 'epidemic' ET12 strain of *B. cepacia* does not produce C14 lipase (Govan *et al*, 1993).

A small proportion of isolates of *B. cepacia*, from CF patients, are non-saccharolytic and such organisms may be misidentified as *Alcaligenes* or *Comomonas*. Saccharolytic and non-saccharolytic strains may possess the same ribotype (Pitt *et al*, 1996).

B. cepacia is an incredibly versatile organism, capable of utilising a wide range of nutrients for growth; these range from simple salts such as ammonium acetate (Geftic *et al*, 1979) to complex organic compounds such as 2,4,5-trichlorophenoxyacetic acid, the principal herbicide in 'agent orange' (Kilbane *et al*, 1983). Aromatic and polyhalogen compounds can be catabolised by *B. cepacia* and poly-β-hyroxyalkanoates accumulated as reserve materials. *B. cepacia* is intrinsically resistant to antimicrobial agents, including potent anti-pseudomonal antibiotics (Gilardi, 1971) and can even utilise penicillin G as the sole carbon, nitrogen and energy source (Beckmen & Lessie, 1979). *B. cepacia* can survive in distilled water, saline and disinfectants for many years (Holmes, 1986; author, unpublished results).

Without the use of selective medium, *B. cepacia* may be difficult to culture from human respiratory secretions, particularly in CF patients colonised with *P. aeruginosa*. Several media have been developed (Gilligan *et al*, 1985; Welch *et al*, 1987; Henry *et al*, 1997): including a commercially produced *B. cepacia* selective medium (Mast Diagnostics). Selective agents are generally antibiotics; such as ticarcillin and polymyxin in Mast *B. cepacia* medium. Unfortunately, other organisms such as *Alcaligenes xylosoxidans*, *Comomonas acidovorans*, and some strains of *P. aeruginosa* may also grow on the Mast selective medium. It is therefore important that presumptive identification of *B. cepacia* be confirmed by the use of multitest identification systems such as API 20NE. If large numbers of isolates are to be investigated these can be screened for characteristic biochemical reactions on

arginine-glucose medium (Govan, 1989) to avoid excessive use of the expensive commercial kits such as API 20NE.

In some cases, identification of isolates of *B. cepacia* can prove difficult, even with the use of commercially available identification kits, and it has been speculated that as many as 10 - 20% of *B. cepacia* isolates fall into this category. Mis-identification of an isolate of *S. maltophilia* as *B. cepacia* has been reported (Burdge *et al*, 1995). Such a mistake may have serious consequences in CF patients. A policy of segregation of CF patients following the initial isolation of *B. cepacia* from sputum culture is generally undertaken to prevent the spread of *B. cepacia* to non-colonised patients. If an isolate is wrongly identified as *B. cepacia* the patient will probably be exposed to *B. cepacia* colonised patients and is likely to acquire a genuine strain of *B. cepacia*.

B. cepacia is often described as ubiquitous, the natural habitats being soil, water and vegetation, in particular the plant rhizosphere. Interestingly, *B. cepacia* can be surprisingly difficult to culture from environmental samples. In a study, involving the use of selective media and 619 samples from environmental sources from homes, soil, water, sink drains, vegetables, food store counters and salad bars *B. cepacia* was cultured from only 4.5% of the samples, and these were confined to salad bars (Fisher *et al*, 1993). Another study involved 55 environmental samples from soil, pond water and vegetation from tropical and temperate greenhouses; despite the use of enrichment and selective media only 12 (17%) isolates of *B. cepacia* were cultured

(Butler *et al*, 1995). These findings indicate that *B. cepacia* is not readily isolated from all environmental sources and is not as ubiquitous as other pseudomonads.

8.3 BIOLOGICAL CONTROL AGENT

Because of nutritional adaptability and its ability to antagonise and suppress soil-borne plant pathogens, there has been growing interest in the use of *B. cepacia* as a biological control agent and bioremediation agent for herbicide-contaminated soils (Homma *et al*, 1989; Hebbar *et al*, 1992; Folson *et al*, 1990). In their natural habitat, *B. cepacia* probably play a useful role in the control of phytopathogenic fungi resulting in enhanced crop yields. Antifungal products produced by *B. cepacia* include pyrrolnitrin and related phenylpyrroles (Bevivino *et al*, 1994). At present, there is great concern that bulk spraying of *B. cepacia* into the environment as a biological control agent may present a serious human hazard for agricultural workers and for immunocompromised individuals, in particular CF patients. Several studies have investigated the properties of clinical and environmental isolates of *B. cepacia* and claimed that the two groups are distinguishable. However, both clinical and environmental isolates have the ability to macerate onions (Butler *et al*, 1995). A report of macerated hyperkeratotic foot lesions or 'foot rot' amongst troops training in swamps in northern Florida demonstrates that an environmental isolate of *B. cepacia* has the ability to cause infection in humans (Taplin *et al*, 1971). A key issue in *B. cepacia* epidemiology and the controversial debate on the human risk of *Burkholderia* biopesticides has been the origins of the genomovar III isolates and the link between environmental and clinical isolates of the *B. cepacia* complex. As

already mentioned, there is now evidence of environmental and clinical isolates of *B. cepacia* belonging to the genomovar III group. Many of these issues were addressed at a specially convened meeting of the United States Environmental Protection Agency (US EPA) on July 20th 1999 (www.epa.gov/pesticides).

8.4 PATHOGENICITY

B. cepacia is virtually non-pathogenic in healthy individuals, however in recent years, pneumonia and septicaemia caused by *B. cepacia* have resulted in fatalities in otherwise healthy individuals (Pujol *et al*, 1992). Generally, *B. cepacia* tends to colonise patients rather than infect them although fatalies can occur, particularly in severely compromised patients.

Prior to the 1980's, infections caused by *B. cepacia* were primarily restricted to hospitalised patients exposed to contaminated disinfectants and anaesthetic solutions and included infections of soft tissue, respiratory, urinary tract and bacteraemia ocassionally associated with endocarditis and septic shock (Phillips & Eijken, 1971; Cabrera & Drake, 1975). Immuno-deficient patients may succumb to *B. cepacia* infections which include endocarditis (particularly in intravenous drug users), peritonitis, meningitis, pneumonia, bacteraemia and infections of the urinary tract, surgical and burn wounds (Goldman & Klinger, 1986). *B. cepacia* can cause serious problems following contamination of healthcare products such as medical devices and solutions used in medical practice (Craven *et al*, 1981; Gilardi, 1983). Use of *B. cepacia* contaminated ear drops were also thought to lead to multiple brain

abscesses secondary to suppurative otitis media in an offshore oil worker (Hobson *et al*, 1995).

The first evidence of transmission of *B. cepacia* from CF patients to a non-CF patient was demonstrated in a previously healthy mother who acquired the 'epidemic' strain from her two CF children who were colonised with the strain. The mother became chronically colonised resulting in bronchiectasis (Ledson *et al*, 1998a).

Nebuliser devices are possible sources of infection in non-CF patients (Takigawa *et al*, 1993). Nebulised albuterol therapy was responsible for an outbreak of *B. cepacia*, involving 42 patients, in an intensive care unit; in the same unit, a medication preservative, benzalkonium chloride, was also found to be contaminated with *B. cepacia* (Hamill *et al*, 1995). *B. cepacia* is also an important pathogen in patients with chronic granulomatous disease (CGD); causing pneumonia, bacteraemia, skin abscesses, cervical adenitis, and disseminated fatal infection (Sanford, 1995; O'Neil *et al*, 1986; Lacy *et al*, 1993). *B. gladioli* can also be a cause of pneumonia, in these patients, and it is important to distinguish this organism from *B. cepacia*. This may prove difficult especially as many of the commercially available identification kits do not differentiate between the two organisms. The role of *B. cepacia* in CF infections will be discussed later in this chapter.

8.5 VIRULENCE FACTORS

In contrast to the extensive armoury exhibited by *P. aeruginosa*, *B. cepacia* appears to produce few known virulence factors. Studies on putative virulence determinants produced by clinical isolates of *B. cepacia* suggest that production may be strain dependant. Several studies have shown that the majority of isolates of *B. cepacia* from CF patients demonstrate production of protease, gelatinase and lipase but no production of elastase or haemolysin (McKevitt & Woods, 1984; Nakazawa *et al*, 1987; Gilligan, 1991; Gessner & Mortensen, 1990). In contrast, Govan & Deretic (1996) indicate that many CF isolates do not produce lipase or protease activity. Unlike *P. aeruginosa*, isolates of *B. cepacia* produce phosopholipase C and a relatively few produce extracellular haemolysins (McKevitt & Woods, 1984; Nakazawa *et al*, 1987) which may be heat-labile or heat-stable (Abe & Nakazawa, 1994). In contrast Vasil *et al* (1990), demonstrated that 40% of strains showed haemolysis with at least one form of erythrocyte when tested against a panel of erythrocytes from various species. This suggests that the detection of haemolysins is dependant upon the source of erythrocytes used. All haemolytic strains produce lecithinase activity but the converse is not the case (Nakazawa *et al*, 1987). The 'epidemic' or ET12 lineage of *B. cepacia*, J2315, produces a haemolysin, when grown in a glucose rich medium with rapid aeration, capable of lysing horse and human erythrocytes (Hutchison *et al*, 1998). Isolates of *B. cepacia* from plants and rhizospheres were found to have greater biochemical activity than clinical isolates (Gonzalez & Vidaver, 1979) although Bevivino *et al* (1994) found isolates from rhizospheres to be defective in proteases.

Other extracellular products considered as virulence factors, which may enhance the pathogenic potential of *B. cepacia*, are siderophores such as pyochelin (Sokol, 1986), its biosynthetic precursor salicylic acid or azurechelin (Visca *et al*, 1993), cyclic hydroxamic acid, cepabactin (Meyer *et al*, 1989) and tetrapeptide derivatives, ornibactins (Stephan *et al*, 1993). Pyochelin production has been associated with morbidity and mortality in CF patients colonised with *B. cepacia*.

The fact that not all strains of *B. cepacia* produce recognised virulence determinants and that studies on purified lipase indicate that this enzyme is not cytotoxic to HeLa cells nor is it toxic when injected into mice (Lonon *et al*, 1988) suggests that these extracellular factors do not play an important role in *B. cepacia* infection. However, protease purified from a clinical isolate, instilled into a rat lung, resulted in bronchopneumonia (McKevitt *et al*, 1989). This pathogenicity in the animal model was confirmed when transgenic CF mice were exposed to *B. cepacia*; 70% of the CF mice succumbed to a more severe broncho-pulmonary infection than the control mice (Davidson *et al*, 1995).

Approximately 60% of *B. cepacia* isolates express peritrichous fimbriae or pili while others possess polar fimbriae or are non-fimbriate. Outer membrane protein (OMP) preparations from fimbriated strains of *B. cepacia* demonstrate the presence of three proteins which are not found in non-fimbriated strains. Some OMPs and fimbriae, in particular a 22 kDa protein, present only in peritrichous fimbriae, mediate adhesion to various surfaces such as buccal, tracheal, urinary epithelial cells, pneumocytes and

mucin (Sajjan & Forstner, 1993). Isolates of *B. cepacia* from CF patients have demonstrated binding to both CF and non-CF respiratory mucin as well as buccal epithelial cells but this binding ability is considerably less than with *P. aeruginosa*. However, another study has reported a low incidence of *B. cepacia* binding (Butler *et al*, 1992). The conflicting reports may be explained by the strains of *B. cepacia* used. Butler *et al* (1992) used strains from different geographical locations, which had been phenotyped and genotyped to verify their clonal independence. In contrast, Sajjan *et al* (1992) used isolates from a single clinic which may have been clonally related. Isolates with the highest binding values tended to correlate with patients with severe illness; the 'epidemic' strain demonstrated binding six-fold greater than any of the other strains tested (Butler *et al*, 1992). This difference in binding ability may explain why some strains of *B. cepacia* are only transient colonisers while others persist.

Respiratory mucin in CF patients is highly sulphated which protects the mucin from bacterial degradation unless the bacteria have mucin-sulphatase activity (Tsai *et al*, 1991). A recent study by Jansen *et al* (1999) demonstrated for the first time that *B. cepacia* and *P. aeruginosa* can desulphate mucin, although, it is likely that adhesion to the mucin molecules is a prerequisite for desulphation. Mucin-sulphatase activity was shown to be associated with whole cells of *B. cepacia* and *P. aeruginosa*, both in clinical and environmental isolates and there was no difference between 'epidemic' and non-epidemic strains of *B. cepacia*. However, the levels of mucin-sulphatase may vary considerably between isolates.

Adhesion to respiratory mucin is thought to be associated with intertwined fibres refered to as cable pili (Sajjan *et al*, 1992; Sajjan & Forstner, 1992). The gene responsible for cable pili, *cbl*A has been detected in the ET12 strain of *B. cepacia* (Sun *et al*, 1995) and was originally thought to be associated with transmission of all 'epidemic' strains. However, further studies revealed cable pili were not present in all 'epidemic' strains, suggesting that other factors are involved (Sun *et al*, 1995). A study of clinical and environmental isolates confirmed at least five different structural classes of pilin fibres (Goldstein *et al*, 1995) which correlate with the source from which the strains, expressing the pili, were isolated. Another property associated with fimbriae is haemagglutination which occurs in some strains of *B. cepacia* (Kuehn *et al*, 1992).

Lipopolysaccharide (LPS) preparations from environmental and clinical isolates of *B. cepacia* and the closely related phytopathogen *B. gladioli* exhibit a higher endotoxic activity and cytokine stimulation, *in vitro*, than LPS from the major CF pathogen *P. aeruginosa* (Shaw *et al*, 1995). This surprising observation confirms the pathogenic potential of *B. cepacia*. Hughes *et al* (1997) described the potent priming activity of *B. cepacia* LPS for neutrophils; increased neutrophil recruitment and respiratory burst reponses in the lung may contribute to inflammation, by release of tissue-damaging proteolytic enzymes. *B. cepacia* LPS was also found to be a powerful priming agent for the respiratory burst in monocytes (Zughaier *et al*, 1999). This response was significantly greater than that produced by *P. aeruginosa* or *S. maltophilia*, although this priming was CD14 dependent. A purified form of the

characteristic melanin-like pigment produced by the 'epidemic' strain of *B. cepacia* was found to be an efficient scavenger of superoxide radicals produced in the respiratory burst (Zughaier *et al*, 1999). This response may allow melanin-producing strains of *B. cepacia* to survive phagocytosis and thus assist in the colonisation of the CF lung. The levels of inflammatory markers such as C-reactive protein, neutrophil elastase-α-1-antiproteinase complex are significantly higher in patients colonised with *B. cepacia* compared to those colonised with *P. aeruginosa*. In addition, the levels of these markers are reduced following aggressive antimicrobial therapy, even although *B. cepacia* is not eliminated (Elborn *et al*, 1994).

The majority of clinical and environmental isolates of *B. cepacia* demonstrate smooth LPS (McKevitt & Woods, 1984) whereas isolates from CF patients may be smooth or rough, with the latter type more commonly found (Nelson *et al*, 1994). All isolates expressing rough LPS were serum sensitive, while isolates expressing smooth LPS exhibited a range of responses (Butler *et al*, 1994). There is no evidence that *B. cepacia* changes from smooth to rough in the CF lung, as is observed with *P. aeruginosa*. In CF patients, the presence of LPS-specific antibodies in the serum (IgG) and sputum (IgA) does not prevent colonisation by *B. cepacia*. Some patients even succumb to septicaemia in the presence of high concentrations of circulating antibodies (Nelson *et al*, 1993). Unlike *P. aeruginosa*, *B. cepacia* rarely produce mucoid colonial forms nor synthesise alginate. There has been one reported case of a mucoid colonial variant in a CF patient (Straus *et al*, 1989) as well as an isolated case

in our laboratory; also from a CF patient. In neither case was the polysaccharide responsible for mucoidy identified as alginate.

8.6 *B. CEPACIA* IN CF PATIENTS

The emergence of *B. cepacia* as a major threat to the CF community has led to anxiety in patients, families, carers and clinicians. The first reported case of pulmonary colonisation with *B. cepacia* in CF was in the early 1970s (Ederer & Matsen, 1972); subsequent reports followed in 1977 from Philadelphia (Lourdes *et al*, 1977) and in 1979 from the west coast of the USA (Blessing *et al*, 1979). It has been suggested that *B. cepacia* is not a pathogen in CF but merely a marker of deteriorating lung disease. However, *B. cepacia* was reported as the cause of pneumonia and septicaemia in a 17 year old CF patient (Rosenstein & Hall, 1980). The incidence of *B. cepacia* colonisation in CF patients in North America, increased considerably in the early 1980's to as high as 45% in one Canadian centre (Nolan *et al*,1982; Isles *et al*, 1984; Thomassen *et al*, 1985) and with approximately one third of these patients succumbing to a rapidly fatal outcome (Gold *et al*, 1983).

The clinical course of *B. cepacia* colonisation is unpredictable and can follow three courses: first, no change in clinical status; second, a gradual decline in lung function, or third, a rapid fatal deterioration (Thomassen *et al*, 1985). Approximately 30% of CF patients succumb to the third course called 'cepacia syndrome', a necrotising pneumonia with fever, bacteraemia, elevation of erythrocyte sedimentation rate and leucocytosis resulting in rapid and fatal clinical deterioration (Isles *et al*, 1984).

Colonisation with *B. cepacia* may accelerate decline in pulmonary function and result in poor prognosis (Tablan *et al*, 1985). Most recently, longitudinal analysis based on the Canadian national database and statistical models has shown that *B. cepacia* (at least the ET12 lineage) increases mortality risk over periods of one to several years (Corey, 1999).

It has been speculated that pulmonary decline may be associated with production of anti-*B. cepacia* antibodies by the host resulting in subsequent immune complex mediated damage to the lung (Nelson *et al*, 1994). The major impact of *B. cepacia* in children with CF is on respiratory function. In a disturbing outbreak of *B. cepacia* infection (later identified as genomovar II; i.e. *B. multivorans*) in a large paediatric CF clinic; children with the highest lung function deteriorated faster than children whose lung function was already poor (Whiteford *et al*, 1995). A number of risk factors are thought to be associated with the acquisition of *B. cepacia*. These include increasing age, underlying severe lung disease, use of aminoglycosides in particular tobramycin, CF siblings colonised with *B. cepacia* and hospitalisation (Tablan *et al*, 1985). Interactions between *B. cepacia* and other infecting organisms, in particular *P. aeruginosa*, may also affect the outcome of CF lung disease (Hughes *et al*, 1997). *P. aeruginosa* colonisation alone has also been considered as a predisposing factor. However, not all patients are colonised with this organism prior to colonisation with *B. cepacia* as was demonstrated in a large outbreak in the Edinburgh area in the early 1990's where almost 40% of CF patients colonised with *B. cepacia* were not colonised with *P. aeruginosa* (Govan *et al*, 1993).

Although *B. cepacia* did not appear to be a problem in CF patients in the UK until the early 1990's, the first fatal case of 'cepacia syndrome' was reported in 1986 (Glass & Govan, 1986). During the 1980's the prevalence of *B. cepacia* in a CF centre in Leeds had also increased from 1% in 1984 to 10% in 1988 (Simmonds *et al*, 1990). Similar increases in prevalence were reported from other CF centres in London (Taylor *et al*, 1992) and Manchester (Gladman *et al*, 1992). As a consequence of increased prevalence and growing evidence of cross-infection from patient-to-patient CF centres in the USA as early as 1986 (Thomassen *et al*, 1986), and in Birmingham in 1988, segregated CF patients colonised with *B. cepacia* from those who were non-colonised. Subsequently, segregation was extended to include summer camps and not surprisingly became the subject of considerable controversy amongst some clinicians and patients (Speert *et al*, 1989). A strict segregation policy was instituted at the 3rd National Conference of the Adult CF Association held in Birmingham in 1991 and expanded in 1993 to limit contact between colonised and non-colonised individuals (Newsletter of Assoc. CF Adults, 1993). Despite segregation, the prevalence of *B. cepacia* among CF patients in Birmingham remained at 8.3% in 1991; furthermore, genotyping suggested that social contact outwith the hospital was responsible (Smith *et al*, 1992). Although segregation of CF patients in hospital resulted in a decline in incidence of cross-infection (Govan *et al*, 1993; Thomassen *et al*, 1985), outbreaks of *B. cepacia* still occured in several CF centres throughout the UK, including Edinburgh and Manchester (Govan *et al*, 1993), Cardiff (Millar-Jones *et al*, 1992), and Liverpool (Smyth *et al*, 1993). Accumulated evidence indicated that a single highly transmissible strain of *B. cepacia* was responsible for inter-

regional spread amongst CF centres in the UK. In 1996, this 'epidemic' strain was found to be present in 50% of the CF centres throughout the UK and accounted for 38% of all *B. cepacia* isolates from patients in the UK (Pitt *et al*, 1996). Meanwhile, molecular epidemiological evidence had shown a clonal relationship between this strain and an epidemic strain responsible for an outbreak in North America (Sun *et al*, 1995). Currently, this highly transmissible strain is referred to as the Edinburgh/Toronto or ET12 lineage (Govan *et al*, 1996). ET12 based on its association with the Edinburgh and Toronto outbreak and its identification by multilocus enzyme electrophoresis as electrophoretic type 12 (Johnson *et al*, 1994).

Colonisation with more than one strain of *B. cepacia* is unusual, occuring in less than 10% patients (Pitt *et al*, 1996). However, several centres have reported episodes when patients initially colonised with non-epidemic strains have later acquired the 'epidemic' strain (Ledson *et al*, 1998b; Webb & Govan, 1998). This super-infection poses a difficult problem in patient management, namely whether patients colonised with the 'epidemic' strain of *B. cepacia* should be further segregated from those colonised with non-epidemic strains.

8.7 EPIDEMIOLOGY

Prior to 1980, epidemiological investigations into infections caused by *B. cepacia* were hampered by the lack of typing systems. During the 1980s, however, a number of phenotypic fingerprinting techniques included biotyping, bacteriocin susceptibility and production, antimicrobial susceptibility and serotyping were developed.

The first typing system reported for 'fingerprinting' isolates of *B. cepacia* was serotyping. A study carried out using 139 isolates of eugonic-oxidizer group1 described five serotypes I, Ia, Ib, II and III. A sixth serotype IV was later described by Klinger *et al* in 1984. A large proportion of CF isolates were type I, compared with only 8% of isolates from other sources, however, many isolates, particularly those from environmental sources, were polyagglutinable or nontypeable.

Monteil's group differentiated seven somatic 'O' and five flagellar 'H' antigens in a collection of 285 *B. cepacia* isolates (Heidt *et al*, 1983); with two new serotypes, in each category, added at a later stage (Werneburg & Monteil, 1989). Typeability was 98% and 43% for 'O' and 'H' antigens, respectively. A Japanese system described 10 serotypes but only 12% of the 105 isolates were typeable by this method (Nakamura *et al*,1986) . Another method used antisera raised against unheated whole bacteria but only 39% of isolates were typeable (McKevitt *et al*, 1987).

Three biotyping schemes were investigated with the first system classifying *B. cepacia* into four biotypes on the basis of growth at 41^{o}C and gelatin liquifaction at 37^{o}C (Esanu & Schubert, 1973). Another system divided *B. cepacia* into eight biovars on the basis of biochemical characteristics including aesculin hydrolysis, nitrate reduction, o-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis and pigment production (Richard *et al*, 1981). The third scheme involved the use of the commercially available identification kit, API 20NE, but results were too variable for it to be of any use as an epidemiological typing system.

Bacteriocin typing was first demonstrated by Gonzalez & Vidaver (1979) and divided 30 strains into two groups on the basis of bacteriocin production. As only 30% of *B. cepacia* produce bacteriocins; bacteriocin production alone is not a suitable method of typing isolates of *B. cepacia*. Govan & Harris (1985) developed a more discriminating method which was based on susceptibility to and production of bacteriocins. This system differentiated 373 isolates of *B. cepacia* into 44 cepaciacins with 95.2% typeability.

In the mid 1990s, a multi-centred comparative study assessed the discriminatory powers of various phenotypic systems including biotyping, bacteriocin susceptibility and production, antimicrobial susceptibility and serotyping was compared with ribotyping (Rabkin *et al*, 1989). The authors concluded that ribotyping, serotyping and biotyping were useful methods for epidemiological fingerprinting isolates of *B. cepacia.* Antimicrobial susceptibility by disk diffusion was extremely variable and not considered to be a useful typing method and although bacteriocin typing was the most sensitive phenotypic system for detecting differences between groups of isolates or unrelated isolates, results varied substantially among outbreak-related strains.

SDS-PAGE and pyrolysis mass spectroscopy (PMS) were applied to 15 isolates of *B. cepacia*, from CF patients, and 11 were indistinguishable by PMS and found to be the 'epidemic' strain, whereas the remaining four isolates were different (Corkill *et al*, 1994). SDS-PAGE was found to be less discriminatory than PMS.

Development of DNA-based genomic fingerprinting systems in the early 1990's provided a greater understanding of the epidemiology of *B. cepacia* and confirmed the existence of 'epidemic' strains (Dasen *et al*, 1994; Kostman *et al*, 1992; Stull *et al*, 1988). Numerous studies were then undertaken in the hope of determining how *B. cepacia* was transfered among CF patients.

Johnson *et al* (1994) used multilocus enzyme electrophoresis (MLEE) and ribotyping to examine 83 isolates of *B. cepacia* cultured from CF patients and from nosocomial and environmental site isolates. MLEE was found to be more discriminating than ribotyping and demonstrated that environmental isolates of *B. cepacia* were genomically different from pulmonary and nosocomial isolates. MLEE also provided evidence that two CF patients from the UK were colonised with the 'epidemic' ET12 lineage of *B. cepacia* at an Ontario summer camp (Johnson *et al*, 1994). MLEE has the disadvantage of being labour intensive and technically too complex for use in most clinical laboratories. This technique has also been applied to isolates of *B. cepacia* from decayed onions, soils, and clinical sources but considerable diversity was demonstrated among the isolates with no evidence of shared clonality in the case of clinical and environmental isolates (Yohalem & Lorbeer, 1994).

Ribotyping of *B. cepacia* has been shown to to have good specificity and sensitivity (Stull *et al*,1988) and numerous epidemiological studies have been undertaken. Early studies provided evidence of clusters of isolates with the same ribotype within

individual CF centres suggesting cross-infection or a common source (LiPuma *et al*, 1988). Wilkinson & Pitt (1995a) found that 35% of isolates of *B. cepacia* from CF patients in the UK produced the same ribotypes, confirming evidence of spread of the ET12 lineage. Transatlantic spread of the ET12 lineage was demonstrated with the use of ribotyping (Johnson *et al*, 1994; Sun *et al*, 1995). In one study, considerable variation in colonial morphology in 14 isolates of *B. cepacia*, recovered from a CF patient over a two and a half year period, were reported. However, ribotyping showed them to be all clonally-related (Larsen *et al*, 1993).

Four PCR techniques have been applied in epidemiological studies of *B. cepacia*; these include arbitrarily primed PCR (AP-PCR), PCR-ribotyping, enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR), and random amplified polymorphic DNA (RAPD).

AP-PCR provided evidence of patient-to-patient transmission of *B. cepacia* in CF patients or alternatively the presence of a common hospital source (Bingen *et al*, 1993). Isolates of *B. cepacia* from patients colonised for periods of 3-14 months were shown to be similar by AP-PCR. This is in contrast to observations of changes occuring in ribotypes and PFGE profiles but not in MLEE types in *B. cepacia* cultured from CF patients over a period of several years (Johnson *et al*, 1994). It was speculated that these alterations might be due to selective pressures *in vivo* and minor genomic base changes.

When compared to ribotyping, AP-PCR was shown to be as discriminating but not as discriminatory as PFGE (Bingen *et al*, 1993). However, Johnson *et al* (1994) argued that AP-PCR lacks reproducibility and is not sufficiently reliable to evaluate clonal diversity of *B. cepacia*. Ellsworth *et al* (1993) found AP-PCR vulnerable to subtle changes in annealing temperature, template and primer concentrations and magnesium concentration, resulting in loss of major bands. When this technique was compared to PCR-ribotyping and ERIC-PCR, greater day-to-day variation in fingerprints was obtained by AP-PCR (Liu *et al*, 1995).

PCR-ribotyping was shown to be as discriminatory as conventional ribotyping, with the advantage of being more rapid to perform and avoiding the use of radioactive material (Kostman *et al*, 1992). Discriminatory powers of PCR-ribotyping could be improved by restriction digestion of the PCR product, however, Mahenthiralingham *et al* (1996) were unable to demonstrate any improvement in discrimination with the use of *Hae*III, in their study. In an earlier study using 23 distinct unrelated isolates of *B. cepacia*, PCR-ribotyping could only identify 12 types, even after digestion of the PCR product using *Alu*I (Liu *et al*, 1995). PCR-ribotyping is reproducible but is less discriminating than PGFE, ERIC-PCR or AP-PCR. PCR-ribotyping has an advantage that only small amounts, as little as 10pg, of template DNA are required to amplify polymorphisms, but this can also be a disadvantage in that trace amounts of contaminating DNA may interfere with banding patterns (Mahenthiralingham *et al*, 1996).

RAPD was able to group epidemiologically related *B. cepacia* which had previously been shown to be related by PFGE and ribotyping (Mahenthiralingham *et al*, 1996). Two conditions which affect reproducibility in RAPD typing are the concentration of DNA template and the concentration of the primer. RAPD is relatively simple to perform and this enables genomic comparison even when large number of isolates are involved. However, RAPD was demonstrated to be less discriminating than PFGE when fingerprinting isolates of *B. cepacia* (Steinbach *et al*, 1994).

A study of *B. cepacia* cultured from 60 CF patients in a CF centre in Verona, Italy and examined by FIGE revealed that 32 (53%) patients were colonised with different strains while the remaining 28 (47%) patients were colonised with isolates belonging to one of 10 distinct genotypes (Cazzola *et al*, 1996). Evidence of direct patient-to-patient transmission was only found in 10 cases including two classmates. Examination of eight pairs of siblings revealed that only three pairs were colonised with the same strain of *B. cepacia*; only one sibling was infected out of a further three pairs and transient infection was observed in the remaining two pairs of siblings.

Genotyping by PFGE showed that most of the isolates of *B. cepacia*, from CF patients in a French centre, were clonally related, suggesting either cross infection or a common source (VuThien *et al*, 1996). The prevalence of 14%, in this centre, was high compared to the French national average of 2%.

8.8 TRANSMISSION OF *B. CEPACIA* IN CF PATIENTS

The first evidence of patient-to patient transmission of *B. cepacia* was demonstrated at a CF educational camp using ribotyping (LiPuma *et al*, 1990). Further compelling evidence for direct transmission between patients included close contact in hospital (Smith *et al*, 1993), and social contact at a fitness class (Govan *et al*, 1996). Numerous studies, using ribotyping, PFGE, MLEE and other genomic fingerprinting systems have confirmed these observations of person-to-person transmission by certain strains of *B. cepacia*, in particular the ET12 lineage (Bingen *et al*, 1993; Smith *et al*, 1993; Govan *et al*, 1993; Corkill *et al*, 1994; Pegues *et al*, 1994; Whiteford *et al*, 1995). Transfer of respiratory secretions, from colonised patients, is considered to be the principal source of *B. cepacia* acquisition among CF patients (Pitt & Govan, 1993; Govan *et al*, 1996). *B. cepacia* has been cultured from the air in a room occupied by a *B. cepacia* positive CF patient and viable organisms found to persist for up to 45 minutes (Humphreys *et al*, 1994). However, other studies have been unable to detect airborne contamination even when sampling the air beside a *B. cepacia* colonised patient undergoing physiotherapy (Govan *et al*, 1993).

No evidence of patient-to patient transmission was found in a study of the UK's largest adult CF centre at the Royal Brompton hospital although nosocomial infection may have occured (Stableforth & Smith, 1994). This may be explained by the fact that patients attending this hospital come from a wide geographical base including overseas, and did not have the same opportunity for social interactions outwith the hospital as may occur in other CF centres. Another study on 65 isolates

from 17 CF patients plus five patients who had undergone transplantation also found no evidence of person-to-person spread (Steinbach *et al*, 1994). This study showed that each patient harboured a different strain of *B. cepacia.* There is also convincing evidence that *B. cepacia* is not invariably transmitted even between CF siblings. Glass & Govan (1986) reported a lack of transfer between a CF sibling, who succumbed to 'cepacia syndrome' following colonisation with a genomovar III *B. cepacia*, and her CF sibling. Similar findings were observed in the Verona study refered to previously. In this study of eight pairs of siblings; only three pairs were chronically infected with the same strain of *B. cepacia* and only one sibling was infected in each of the other five pairs (Cazzola *et al*, 1996). In the early 1990's, a growing suspicion that transmissibility is strain dependant was confirmed in a patient who harboured two strains of *B. cepacia*; one being the 'epidemic' strain. Following a close relationship only the 'epidemic' strain was transmitted to the patient's girlfriend (Govan *et al*, 1993).

Indirect transmission of *B. cepacia* may occur since patients contaminate environmental fomites such as drinking vessels, respiratory equipment and disinfectants which can then act as a source of infection for other patients (Burdge *et al*, 1993; Nelson *et al*, 1991). Nebulisers are a potential but apparently not a major source of acquisition of *B. cepacia* as *B. cepacia* was recovered from only three of the 35 home-use nebulisers investigated (Hutchinson *et al*, 1996). PFGE demonstrated that one of the two strains recovered from a nebuliser was the same as the isolate obtained from the patient's sputum. In the other two cases, *B. cepacia* was

recovered from nebulisers but no *B. cepacia* was detected in sputum culture from the patients, suggesting that the isolates from the nebulisers were probably obtained from an environmental source.

A final intriguing question remains. Proponents of the use of *B. cepacia* biopesticides have claimed that there is no scientific evidence that environmental strains are responsible for colonising CF patients. In a study of environmental samples, only four isolates of *B. cepacia* were recovered from 58 samples and when these isolates were compared to those isolated from CF patients, ribotyping demonstrated that the environmental isolates were distinct from any of the clinical isolates (Honicky *et al*, 1993).

8.9 AIMS

B. cepacia is a cause of great concern within the CF community. Anxiety arises, in particular, because of evidence of patient-to-patient spread of transmissible strains, and the association of certain strains and genomovars with the lethal outcome known as 'cepacia' syndrome. A reliable discriminatory typing system for *B. cepacia* is essential for ongoing surveillance studies and for prospective studies to identify reservoirs and modes of spread. As PFGE is regarded as the 'gold standard' in molecular typing techniques, the aim of this section of the thesis was to assess the use of PFGE for fingerprinting *B. cepacia* using the recognised criteria for an ideal typing system. According to Hawkey (1989), an ideal typing system should:
1. Possess the ability to type the majority of strains encountered: 2. Demonstrate

good discriminatory powers and reproducibility: 3. Be readily applicable to environmental and clinical isolates as well as laboratory collections of strains: 4. Be easy to perform and inexpensive. Another aim was to compare PFGE with another genomic typing system, PCR-ribotyping, and with a simpler and faster phenotypic method, bacteriocin typing.

The first UK isolate of the 'epidemic' strain of *B. cepacia*, later known as ET12, was obtained from an Edinburgh CF patient in August 1989. For this thesis, a longitudinal study was made of the isolates of *B. cepacia* recovered from this patient over the nine year period to determine whether PFGE could detect any variation in the isolates as the organism adapted to the lung environment. A parallel study investigated whether alterations in PFGE patterns occured as the strain spread amongst the Edinburgh CF population.

Following application to North America regulatory authorities for bulk environmental release of *B. cepacia* as a commercial prospect and soil remediator, a further aim of the thesis was to use PFGE to investigate the relationship between environmental *B. cepacia* and those colonising CF patients.

8.10 RESULTS

8.10.1 Genomic fingerprinting of *B. cepacia* by PFGE

Chromosomal DNA was extracted frrom isolates of *B. cepacia* using the method described by Vasil *et al* (1990) except that the concentration of low melt agarose,

used for making the agarose plugs, was reduced from 2% to 1% to provide clearer banding patterns.

Digested chromosomal DNA was separated by PFGE using inital and final pulse times of 2.9 s and 35.4 s, respectively for 20 h at 14^{o}C. These conditions were provided by Bio-Rad from information given to them regarding the estimated range in size of the DNA fragments produced following digestion with restriction enzymes *Xba*I and *Spe*I. This technique worked well for about two years and then failed to produce banding patterns clear enough to allow comparison of isolates of *B. cepacia*. As the technique had not altered in any way, it was decided to investigate the chemicals involved. The outcome was that EDTA, used in the making of buffers, gave variable results depending on the manufacturer; EDTA supplied by Bio-Rad producing clearer PFGE profiles than that of EDTA obtained from Sigma. Further investigation into the technique used for lysing *B. cepacia* revealed that superior results were achieved by substituting proteinase K with 0.5% Triton X-100 in the lysis buffer. Unfortunately, this procedure failed to work for the ET12 lineage of *B. cepacia*. The 'ET12' problem was overcome by incorporating an additional incubation step using lysis buffer containing proteinase K (50 μg/ml) at 55^{o}C overnight following the initial lysis in lysis buffer containing 0.5% Triton X-100. Initially, it was thought that the failure of the ET12 isolates of *B. cepacia* to lyse might be due to the fact that the lineage exhibits rough lipopolysaccharide (LPS). However, no problems were encountered using the 0.5% TritonX-100 lysis

procedure for the extraction of chromosomal DNA from other strains of *B. cepacia* (C1394, C1632, ATCC 17762) also known to have rough LPS (Simpson *et al*, 1994).

To reduce the time taken for PFGE of *B. cepacia*, the initial overnight bacterial culture was shortened by growing the bacteria in 5 ml NBYE for 3 h at 37^{o}C in shaking incubator. One millilitre of the culture was centrifuged at 13,000 rpm for 2 min, washed in 1 ml SE buffer, centrifuged at 13,000 rpm for 2 min and the pellet resuspended in 0.5 ml SE buffer. The plugs were made by adding an equal volume of 1% low melt agarose and pipetting the mixture into the plug mold. The plugs were then lysed by the procedure mentioned above.

8.10.2 Is PFGE a suitable typing system for *B. cepacia* ?

Isolates of *B. cepacia* were used to investigate the major criteria necessary for a good typing system; discriminatory power, reproducibility and stability. With the exception of closely related isolates, considerable diversity in DNA banding profiles was observed when clinical or environmental isolates of *B. cepacia* were examined by PFGE. Fig 8.1 illustrates the degree of discrimination of this technique for nine isolates of *B. cepacia* following digestion with *Xba*I.

Reproducibility of PFGE of *B. cepacia* was demonstrated by making agarose plugs from nine different cultures of *B. cepacia* isolate A599. The plugs were lysed individually, prior to digestion with restriction enzyme *Xba*I. Identical PFGE profiles were illustrated in Fig 8.2.

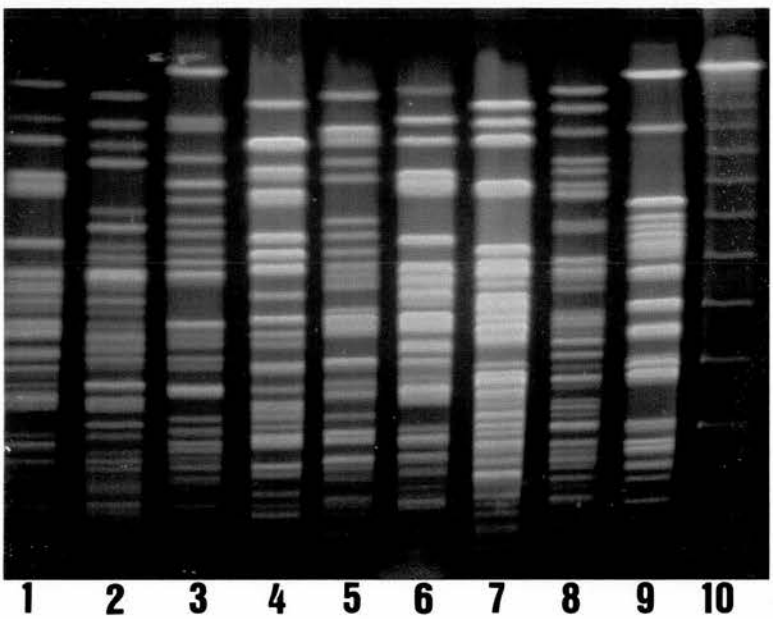


FIG. 8.1. *Xba*I digestion of DNA from nine different isolates of *B. cepacia* showing a range of PFGE profiles. Lanes 1 to 9, J2315, J2553, A552, C1504, A548, C1372, A599, J2543, C1513; lane 10, lambda concatemers (size range 48.5kb to 582 kb, in increments of 48.5kb).

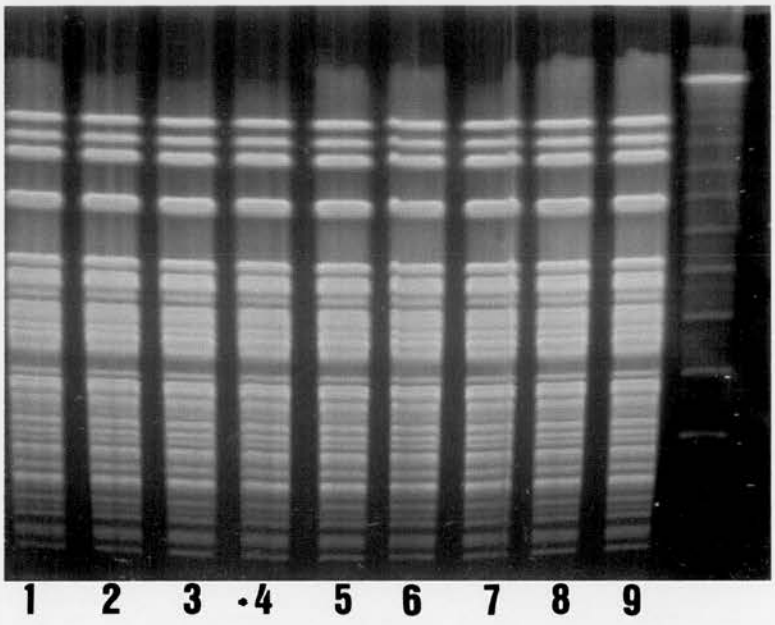


FIG. 8.2. PFGE of *Xba*I digested genomic DNA from nine different preparations of *B. cepacia* isolate A599 (lanes 1 to 9), demonstrating the reproducibility of this technique. Lane 10, lambda concatemers (size range 48.5kb to 582kb, in increments of 48.5kb).

A considerable advantage of PFGE over other typing systems is that a banding pattern, produced by an isolate, can be compared to the patterns produced by other isolates within the same gel or in different gels.

Several isolates of *B. cepacia* were used to investigate the stability of PFGE. Results using the 'ET12' strain of *B. cepacia* (J2315) are illustrated in Fig 8.3. Stability of PFGE for *B. cepacia* was demonstrated both *in vivo* and *in vitro*. J2315, the original UK index isolate stored at -70^{o}C for nine years produced the same pattern as a fresh isolate from the same patient (results not shown).

Passaging of J2315 through numerous subcultures (six passages) on nutrient agar did not alter the PFGE banding profiles nor did culture on the following media; NBYE, Mueller Hinton broth, LB broth, Brain heart infusion broth and Malka medium, prior to DNA preparation have any affect on the PFGE profiles produced. Comparison of DNA preparations made from cultures grown overnight with log phase cultures also showed no difference in the banding patterns produced by PFGE (results not shown).

When J2315 and other strains of *B. cepacia* were inoculated into distilled water or saline, and kept at room temperature, they remained viable for the four year period of this study. During this time, the PFGE profiles remained identical to that of the original isolate J2315. In conclusion, PFGE profiles of *B. cepacia* remain unaltered following storage at different temperatures, starvation in distilled water and saline and culturing in different media prior to extraction of chromosomal DNA.

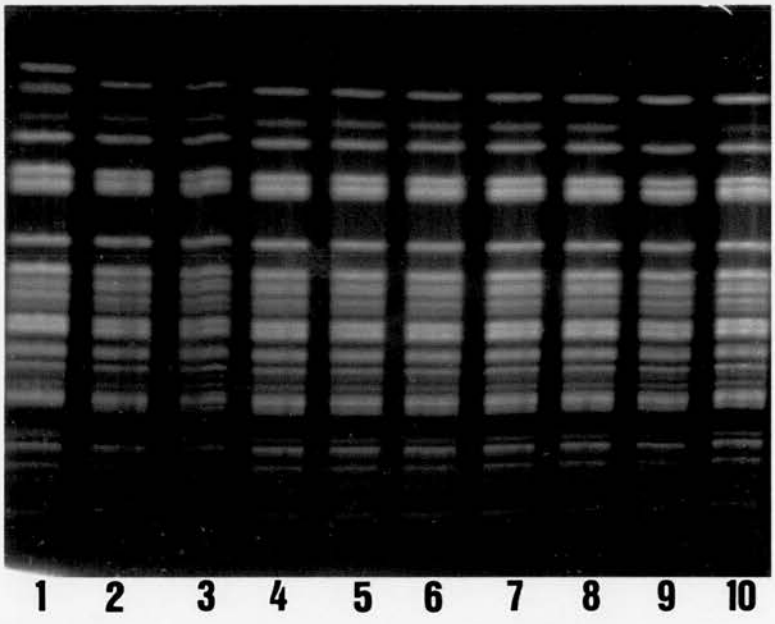


FIG. 8.3. PFGE of *Xba*I digested genomic DNA from *B. cepacia* isolate J2315, under varying conditions. Lane 1, recent fresh isolate (recovered 9 years after original isolate); lane 2, storage at -70^{o}C; lane 3, passaged on 6 occasions; lane 4, grown in NBYE; lane 5, grown in Mueller Hinton broth; lane 6, grown in LB broth; lane 7, grown in Brain Heart Infusion broth; lane 8, grown in Malka medium; lane 9 and 10, starvation in distilled water and saline, respectively.

Longitudinal studies of the first Edinburgh CF patient to acquire the 'epidemic' strain of *B. cepacia* were undertaken over the nine years in which this patient has remained colonised. The PFGE profile of the original isolate J2315, recovered from sputum culture in 1989, was compared to subsequent isolates from this patient; in all cases, the restriction enzyme *Xba*I was used to digest chromosomal DNA. No difference in the PFGE profiles was detected until 1995 when five isolates from this patient were obtained during the gene therapy trials. At this time, two different profiles were observed. Three isolates revealed a three band difference from the original; this change involved the appearance of two new bands and a band missing. The other two isolates produced patterns identical to the original 1989 isolate of *B. cepacia*. In 1996, the PFGE profile of a recent isolate only showed a single band difference involving the appearance of an extra band.

An isolate of *B. cepacia* recovered in 1997 showed an identical PFGE profile to that of the original isolate while the most recent isolate, recovered in 1998 produced a three band difference in PFGE pattern; this change involved the appearance of two new bands and a band missing. A comparison of the PFGE profiles of the most recent isolate taken in 1998 and the original can be seen in lanes 1 and 2 in Fig.8.3. All differences in the PFGE profiles occured in the top region of the agarose gel, suggesting variation in the larger fragments of DNA.

Interestingly, isolates of *B. cepacia*, which demonstrated a difference in PFGE profile using restriction enzyme *Xba*I, showed no change in their PFGE profiles following digestion with restriction enzyme *Spe*I (results not shown).

The results of this study indicate that genomic fingerprinting of *B. cepacia* by PFGE provides good discrimination, reproducibility and stability. One of the disadvantages of this system, however, is that it is time-consuming and typically takes 3 - 4 days before a result can be obtained. With this in mind, it was decided to investigate a more rapid molecular typing system, namely PCR-ribotyping. Another PCR-based random amplified polymorphic DNA (RAPD) typing system was also considered. However, after numerous failures to apply this system satisfactorily to *B. cepacia*, it was abandoned in favour of PCR-ribotyping.

8.10.3 PCR-ribotyping

The reproducibility of PCR-ribotyping for *B. cepacia* was investigated as follows: DNA was extracted from cultures from two different CF patients on six occasions and amplified using PCR-ribotyping. The PCR product was then analysed by electrophoresis and the results, illustrated in Fig 8.4, confirm the reproducibility of the technique. However, variations in banding patterns were observed. These variations occured when DNA extracted from the same strain of *B. cepacia* on different occasions, was amplified by PCR-ribotyping at different times and the PCR products analysed by electrophoresis on different gels (results not shown).

The discriminatory power of PCR-ribotyping was investigated using the same nine strains of *B. cepacia* which were used to demonstrate genomic diversity by PFGE. Comparison of the profiles produced by PCR-ribotyping showed each isolate to be different with the exception of isolates A548 and A599 (lanes 5 and 7, Fig.8.5) which showed very little, if any, differences in their profiles. Since all of the isolates had been shown to be clearly different by PFGE, it seemed that PCR-ribotyping was reasonably discriminatory but not as discriminatory as PFGE.

8.10.4 Comparison of bacteriocin typing, PFGE and PCR-ribotyping

Bacteriocin typing is a reasonably reliable method for typing *B. cepacia*; for example, the ET12 strain invariably produces a bacteriocin type, S3/P0 or S13/P0 pattern. There is only a single indicator difference between the two typing patterns and in general, variation only occurs following storage at -70^{o}C. Another 'epidemic' strain of *B. cepacia*, responsible for a major outbreak in Manchester, produces a bacteriocin type S3/P6. This fingerprint is also very reproducible. Unfortunately, bacteriocin typing failed the discriminating test as a large proportion of isolates of *B. cepacia* produce a bacteriocin type S22/P0 pattern. When these isolates are typed by PFGE, the majority produce unique profiles indicating that they are clonally unrelated.

Interestingly, initial studies using bacteriocin typing suggested that a large group of CF patients attending a Glasgow CF centre were colonised with the same strain of *B. cepacia* (Whiteford *et al*, 1995).

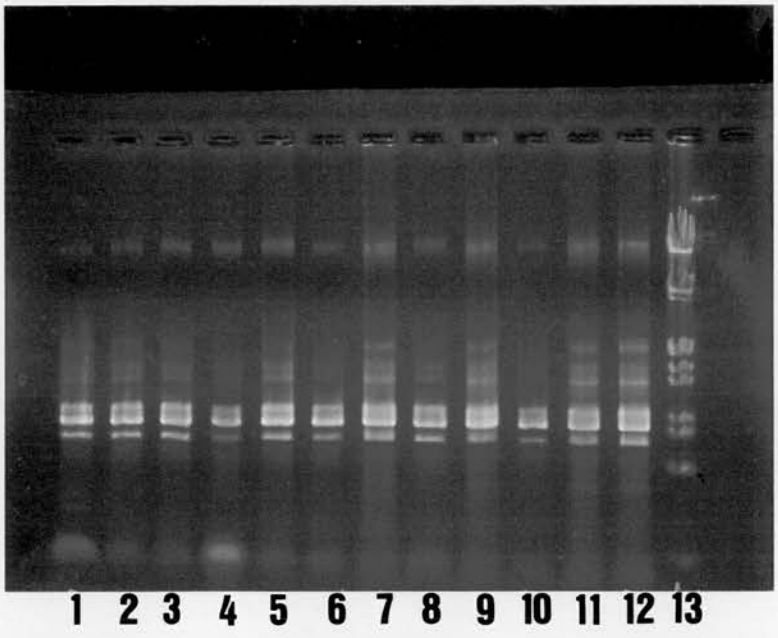


FIG. 8.4. PCR amplification patterns of six different DNA extractions from two different isolates of *B. cepacia* J2315 (lanes 1 to 6) and C1355 (lanes 7 to 12), demonstrating reproducibilty; lane 13 lambda DNA marker (125 to 21,226bp).

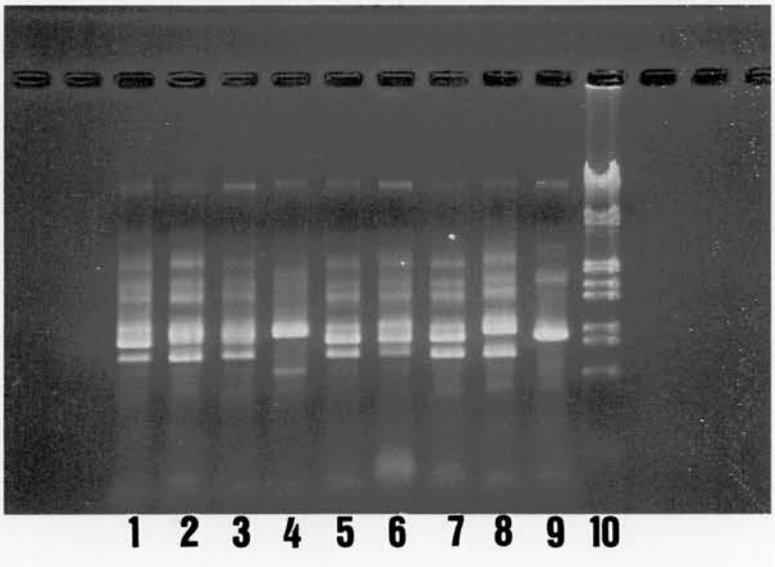


FIG.8.5. PCR amplification of nine different isolates of *B. cepacia.* Lanes 1 to 9, J2315, J2553, A552, C1504, A548, C1372, A599, J2543, and C1513; lane 10 lambda DNA marker (125 to 21,266 bp).

All isolates produced a bacteriocin type S22/P0 pattern and this 'phenotypic' clonality was confirmed when the same isolates were analysed by PFGE.

Eight epidemiologically unrelated isolates of *B. cepacia*, all bacteriocin type S22/P0, were typed by PFGE and PCR-ribotyping to compare the three systems (Figs. 8.6 & 8.7). PFGE analysis revealed that two isolates, C1503 and C1462, showed identical PFGE profiles; isolates C1572 and C2080 showed a two band difference, indicating that they are closely related, and the remaining isolates produced unique profiles suggesting that they are different strains of *B. cepacia*. PCR-ribotyping showed that isolates C1571 and C1596 were obviously different while isolates C1462, C1572 and C2080 produced identical profiles.

Although C1503 produced an identical PFGE profile to that of C1462, the profile produced by PCR-ribotyping revealed a difference in thickness of the dense band and also the absence of a faint band. These minor differences in isolate C1503 suggest that this isolate is different from C1462. The two isolates of *B. cepacia* (C1447 and C1552) show varying degrees of similarity to the three isolates (C1462, C1572 and C2080) with identical patterns by PCR-ribotyping; thus it was difficult to determine whether the strains were the same or different by this technique.

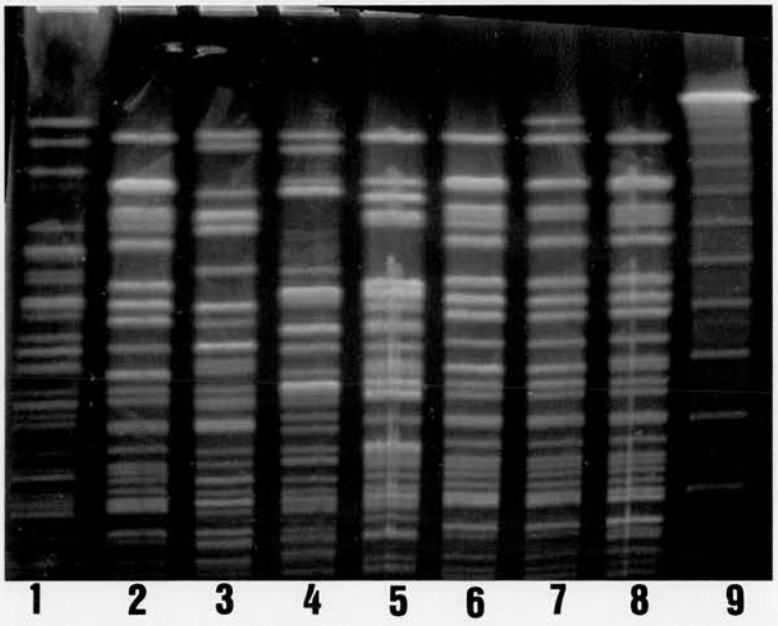


FIG. 8.6. *Xba*I digestion of DNA from eight isolates of *B. cepacia*, all bacteriocin type S22/P0. Lanes 1 to 8, C1447, C1503, C1552, C1571, C1596, C1462, C1572, C2080; lane 9, lambda concatemers (size range 48.5kb to 582kb, in increments of 48.5kb).

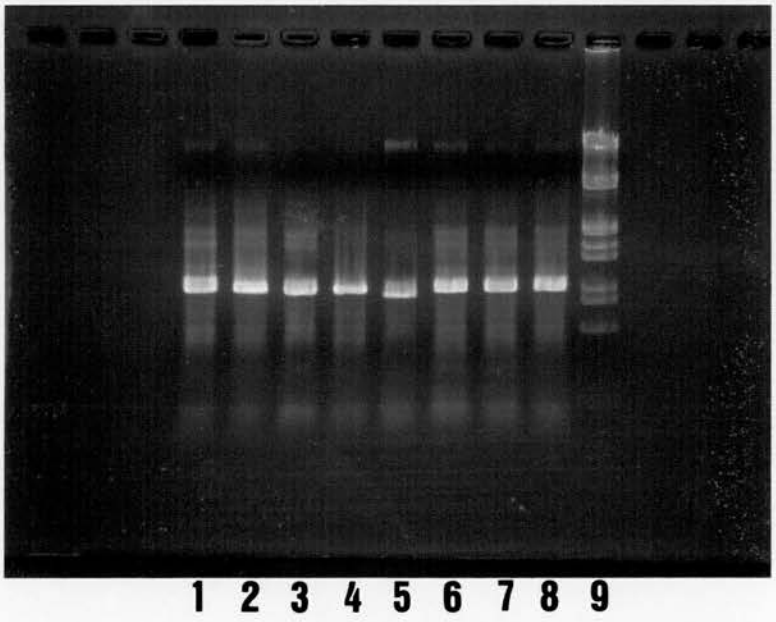


FIG.8.7. PCR-ribotyping of the same eight isolates of *B. cepacia*. Lanes 1 to 8, as above; lane 9, lambda DNA marker (125 to 21,226 bp).

8.10.5 Comparison of PFGE and PCR-ribotyping

Thirteen CF isolates of *B. cepacia* C2701 - C2713, from Verona, Italy were fingerprinted by PFGE and PCR-ribotyping (Figs. 8.8 & 8.9). PCR-ribotyping revealed three groups of patients, each with their own strain of *B. cepacia*. Group 1 comprised of isolates C2701 and C2702, group 2 - C2703 and C2704, and group 3 - C2705, C2707, C2710, C2711, and C2712. The banding patterns produced by the remaining seven patients were each unique, suggesting that these patients were colonised with different strains of *B. cepacia*. These results were confirmed by PFGE although minor differences in PFGE profiles, within the groups, were observed. The PFGE profile produced by isolate C2707 showed a six band difference compared to the banding patterns produced by the other isolates in this group. However, according to the criteria proposed by Tenover *et al* (1995) for interpretation of PFGE profiles, isolate C2707 would be regarded as being related to the other isolates of *B. cepacia* in group 3.

8.10.6 ET12 lineage of *B. cepacia* in Edinburgh CF patients.

The major 'epidemic' strain of *B. cepacia* known as ET12 colonised 15 CF patients in Edinburgh following its emergence as the index isolate, J2315, in August 1989. Figures 8.10 & 8.11 show the slight variations in the PFGE profiles of this strain as it transferred from patient-to-patient. Investigations into the mode of transmission of ET12 among the Edinburgh CF patients provided compelling evidence that social contact was responsible for spread in all cases except one; this patient did not appear to have had contact with any of the other *B. cepacia* colonised patients.

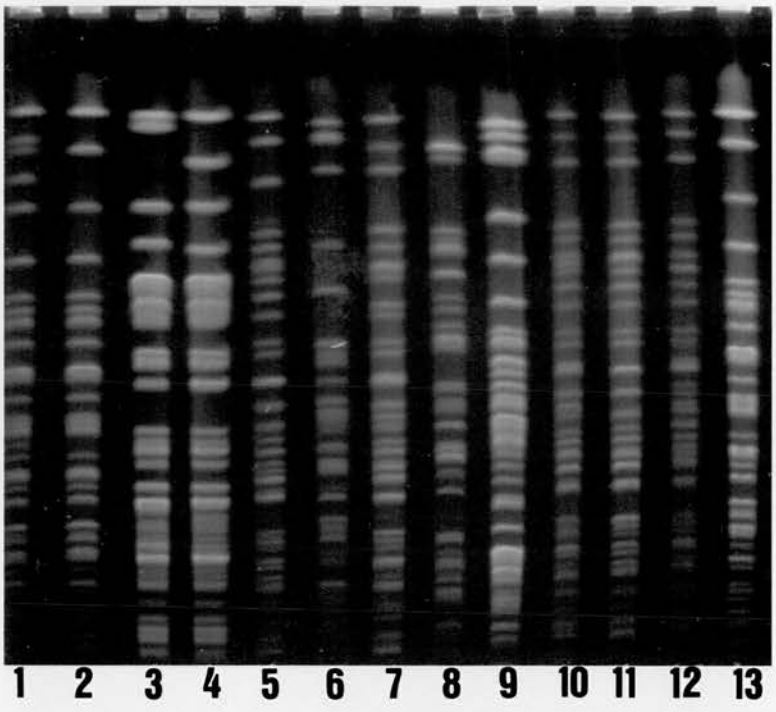


FIG.8.8. *Xba*I digestion of 13 isolates of *B. cepacia* from Verona followed by PFGE. Lanes 1 to 13, C2701 - C2713, respectively.

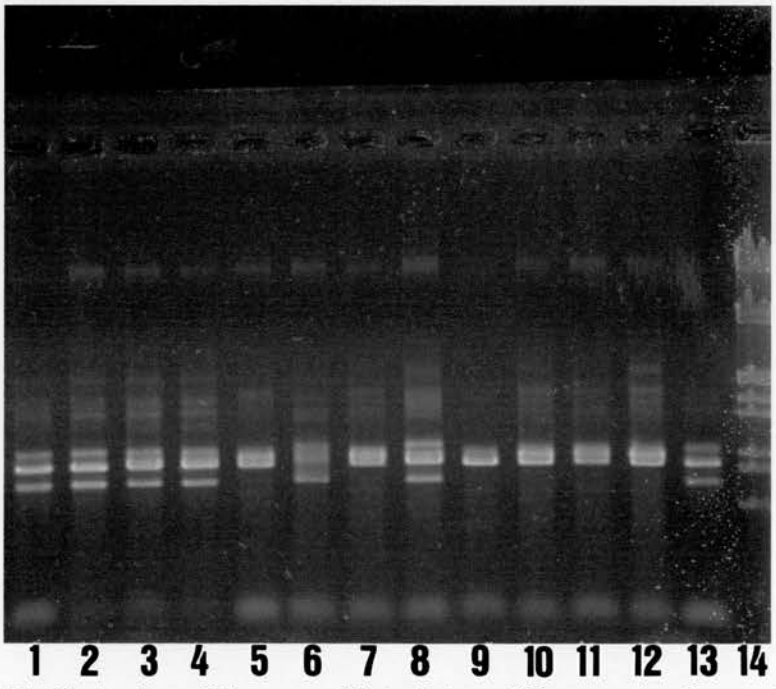
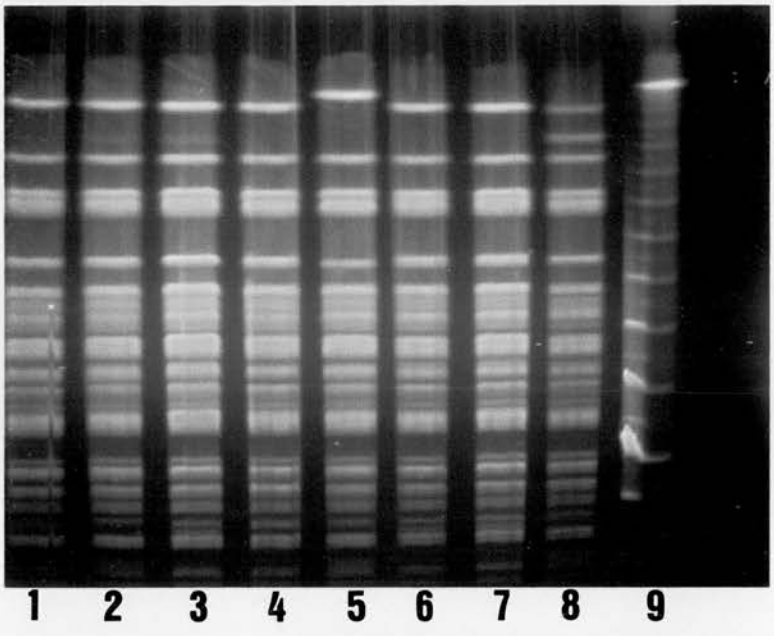


FIG. 8.9. PCR-ribotyping of the same 13 isolates of *B. cepacia.* Lanes 1 to 13, as above; lane 14, lambda DNA marker (125 to 21,226 bp).



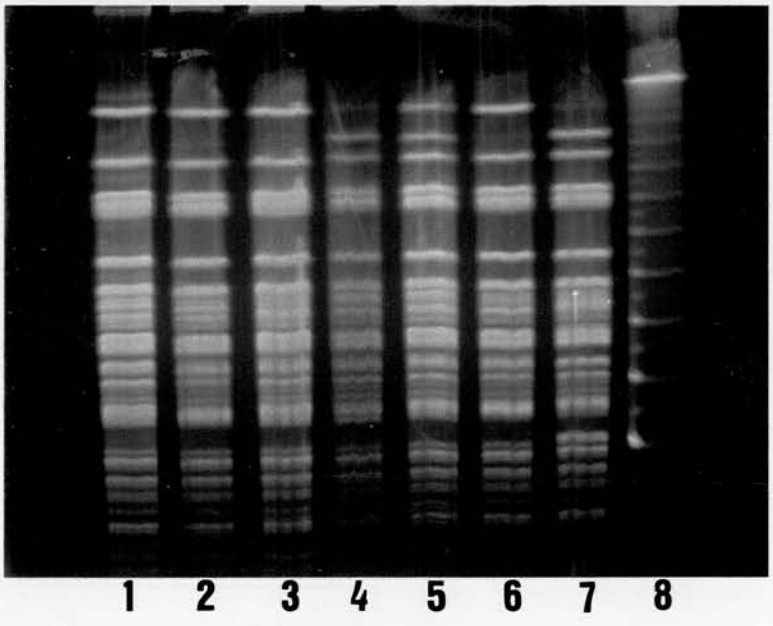


FIG. 8.10 (top) & 8.11 (bottom). *Xba* I digestion of the 15 isolates of the 'epidemic' strain of *B. cepacia* from the Edinburgh CF patients. Fig. 8.10. Lanes 1 to 8, C1355, C1358, C1359, C1382, C1392, C1512, C1525, C1895; lane 9, lambda concatemers (size range 48.5kb to 582kb, in increments of 48.5kb). FIG. 8.11. Lanes 1 to 7, J2315, C1385, C1430, C1372, C1500, C2374, C2606; lane 8, lambda concatemers.

Social contacts included attending the same gym class, drinking partners, boyfriend-girlfriend relationship, a car journey with a colonised patient, and a single visit of a previously non-colonised patient to his *B. cepacia* colonised friend in hospital. The same fifteen isolates of ET12 were typed by PCR-ribotyping (Fig. 8.12) and provided identical banding patterns apart from isolate C1525 in lane 12, which demonstrated an extra band. It would appear, from previous analysis of PCR-ribotyping profiles, that a single band difference, when comparing two PCR-ribotyping profiles, is sufficient to indicate that isolates are different. Thus, the PCR-ribotyping results suggested that isolate C1525 could be different to that of the ET12 lineage of *B. cepacia*. However, the PFGE profile of C1525 was found to be identical to the PFGE profile of J2315. The other PRC-ribotyping results were in agreement with the results of PFGE profiles which demonstrated only minor band differences in the profiles of 5 of the 15 CF patients.

This study provided little evidence to suggest that the Edinburgh version of the *B. cepacia* ET12 lineage has changed to any extent over the eight year period in which this strain has spread amongst the Edinburgh CF community. A comparison of two PFGE profiles produced from the index isolate J2315 and the most recently colonised CF patient (C2606), showed a four band difference, using restriction enzyme *Xba*I (Fig. 8.10). The differences observed included an additional band in isolate C2606, while the other three bands showed only an alteration in density or thickness in comparison to that produced by J2315.

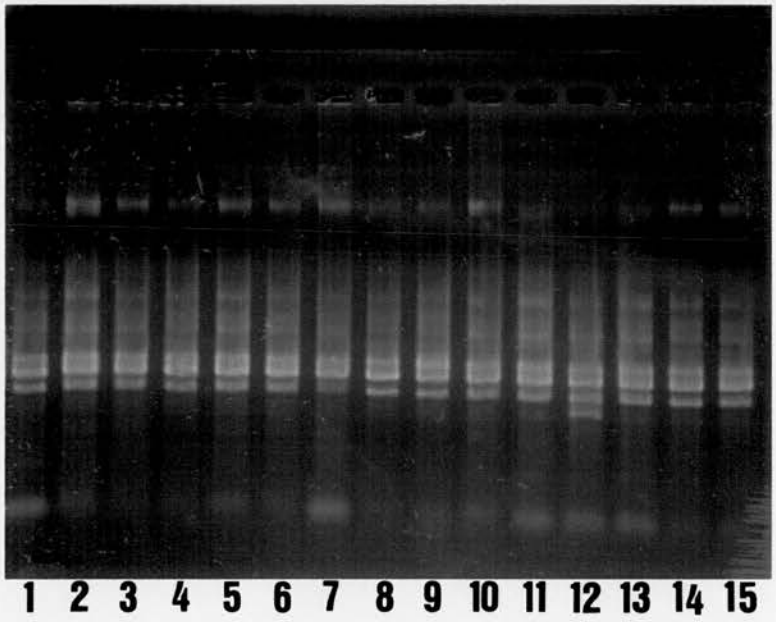


FIG.8.12. PCR-ribotyping of the 15 isolates of the ET12 strain of *B. cepacia*, from the CF patients in Edinburgh. Lanes 1 to 15, J2315, C1355, C1358, C1359, C1372, C1382, C1385, C1392, C1430, C1500, C1512, C1525, C1895, C2606, C2374.

When the restriction enzyme *Spe*I was used to cut chromosomal DNA from *B. cepacia* ET12 isolates from seven of the CF patients, minor differences were again observed in the PFGE profiles (Fig. 8.13). Comparison of PFGE profiles of the index isolate J2315 and isolate C2606, using restriction enzyme *Spe*I, revealed only a slight difference; namely, the appearance of an additional band and difference in intensity of another band.

8.10.7 Genomovars

Recently, organisms presumptively identified as *B. cepacia* have been shown to comprise of five distinct genomic species or genomovar groups, I - V. These are identified as genomovars I - V, with genomovar II and IV renamed as *B. multivorans* and *B. vietnamiensis*, respectively. The group as a whole are refered to as the *B. cepacia* complex.

Although isolates of *B. cepacia* from CF patients have been found in each of the genomovar groups, a large proportion of CF isolates, including the ET12 strain, belong to group III. Nine CF isolates of *B. cepacia*, belonging to genomovar group III were examined by PFGE to determine the relationship, if any, between isolates of *B. cepacia* within this closely-related taxonomic group. PFGE showed genomic heterogeneity even within genomovar group III (Fig 8.14), and similar heterogeneity in PFGE pattern was found in each of the other genomovars (results not shown). The genomovar III isolates investigated consist of an interesting group of strains.

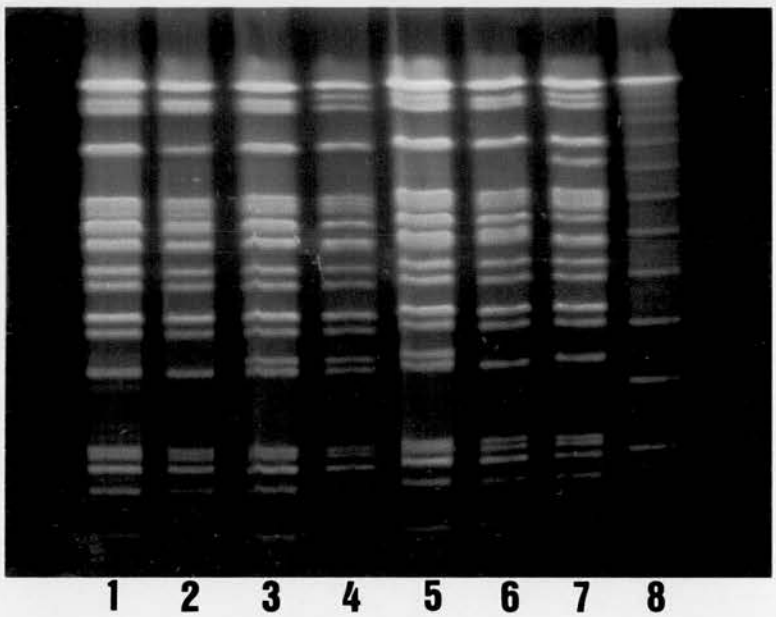


FIG. 8.13. *Spe*I digestion of the same seven isolates of the ET12 strain of *B. cepacia* as in Fig. 8 11. Lanes 1 to 7, J2315, C1385, C1430, C1372, C1500, C2374, C2602; lane 8, lambda concatemers (size range 48.5kb to 582kb, in increments of 48.5kb).

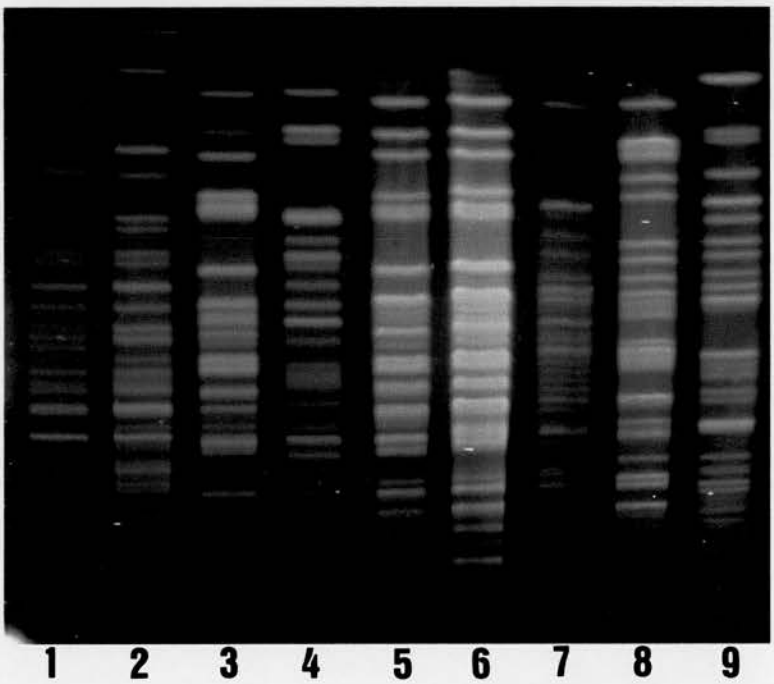


FIG. 8.14. *Xba*I digestion of nine isolates of *B. cepacia* belonging to genomovar III. Lanes 1 to 9, J415, C1335, J2315, C1394, C1773, C1632, J2660, A548, and A552.

The isolate from patient KD (J419), in lane 1, was the first reported case of a CF patient succumbing to 'cepacia' syndrome in the UK in 1986. Surprisingly, *B. cepacia* was not detected in her sister's sputum (C1335) until 4 years after KD's death and interestingly, both isolates showed similar PFGE profiles (lanes 1 & 2). Representives of ET12 and other 'epidemic' strains of *B. cepacia* are shown in lane 3 (ET12 strain), and lane 4 (Manchester 'epidemic' strain). Lanes 5 and 6 show a clonal relationship in isolates from CF patients in Papworth and Newcastle, respectively. Lane 7 is a CF isolate from Cardiff and lanes 8 & 9 are isolates from paediatric CF patients in Edinburgh not associated with the Edinburgh ET12 lineage. Interestingly, the majority of paediatric CF patients in Edinburgh are colonised with their own unique strain of *B. cepacia.*

8.10.8 Evidence of clonality between an environmental and a clinical isolate of *B. cepacia*

Twelve environmental isolates of *B. cepacia* and 12 ATCC type strains were examined by PFGE and the resulting profiles compared to profiles produced by a large number of clinical isolates. Interestingly, an isolate of *B. cepacia* (C2808) recovered from a CF patient produced a striking bright yellow pigmentation when grown on NA medium (Fig. 8.15a). This characteristic is unusual in clinical isolates and is generally confined to environmental isolates of *B. cepacia*. Further investigations revealed that both the clinical isolate and the yellow-pigmented type strain ATCC 25416, isolated from an onion, shared identical biochemical profiles when identification as *B. cepacia* was confirmed by the API 20NE system. In terms

of antibiotic susceptibility, both isolates also produced similar MIC's of ceftazidime (1 mg/l), ciprofloxacin (0.5 mg/l), colomycin (>64 mg/l), trimethoprim (1 mg/l), meropenem (0.25 mg/l), imipenem (4 mg/l), and trobramycin (8 mg/l).

Although different types of rot in onions, including wet and dry rot, have been produced by clinical and environmental isolates of *B. cepacia* both of the isolates C2808 and ATCC 25416 demonstrated the same wet, slimy type of rot after 6 days incubation at 30^{o}C. Further evidence to suggest that these isolates were clonally related was obtained by PFGE, using two different restriction enzymes *Xba*I and *Spe*I, where both isolates produced identical PFGE profiles with each enzyme (Fig. 8.15). Analyses of whole-cell protein electrophoresis patterns also showed that both isolates belonged to genomovar I (results provided by P. Vandamme, Belgium).

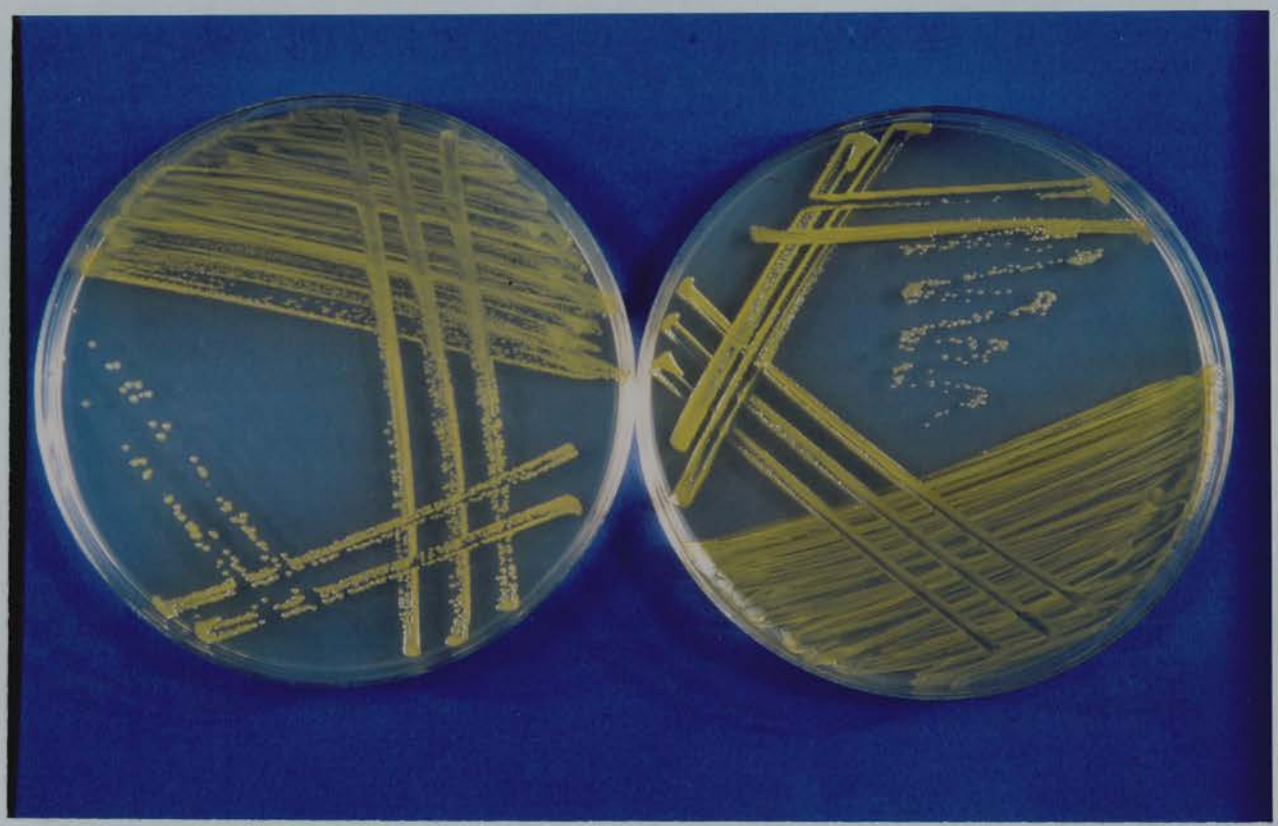


FIG. 8.15a. Clinical isolate C2808 and environmental type strain ATCC 25416 of *B. cepacia* demonstrating the striking bright yellow pigmentation.

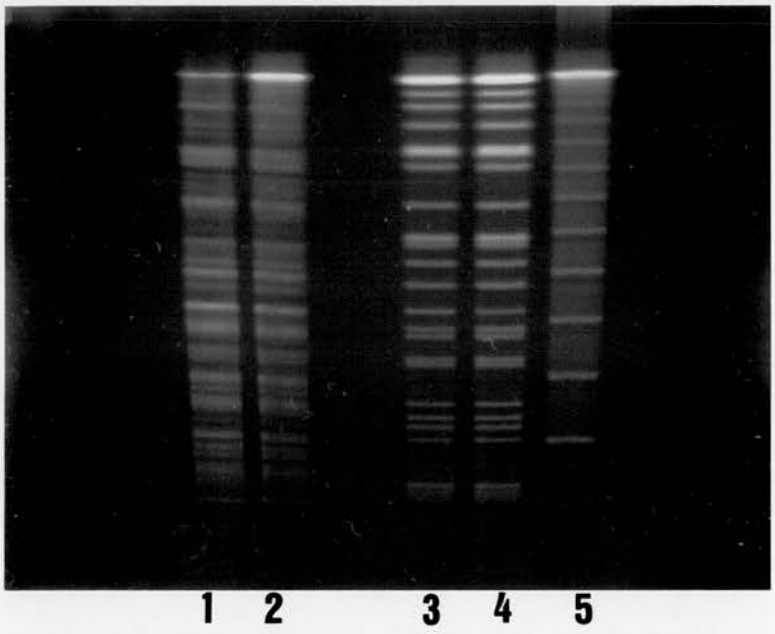


FIG. 8.15. PFGE profiles of environmental and clincal isolates of *B. cepacia*, using restriction enzymes *Xba*I and *Spe*I, respectively. Lanes 1 and 3 C2808; lanes 2 and 4 ATCC 25416; lane 5, lambda concatemers (size range 48.5kb to 582kb, in increments of 48.5kb).

8.11 DISCUSSION

8.11.1 Improvements in DNA extraction methods prior to separation by PFGE

As with all molecular procedures, PFGE can produce variable results with respect to the clarity of the banding patterns produced by isolates of *B. cepacia*. Initially, the DNA extraction method using lysis buffer containing proteinase K appeared to work as clear PFGE profiles were seen in the majority of the agarose gels. However, after a period of time and for no apparent reason the banding patterns produced by PFGE showed faint, ill-defined bands, making comparison of profiles extremely difficult. Having ruled out all other possibilities, these results necessitated the development of another procedure for DNA extraction. The use of Triton X-100 incorporated into the lysis buffer, as a substitute for proteinase K, resulted in consistently clear, well defined profiles showing good resolution. This modification made interpretation of the results and the comparison of PFGE profiles much easier.

Interestingly, the 'epidemic' strain of *B. cepacia* known as ET12 appears to be the only strain which resists lysis by the Triton X-100 method. Initially, the rough LPS structure of ET12 was thought to be a possible explanation for its failure to lyse by this method. However, DNA was successfully extracted from other rough strains of *B. cepacia* using the Triton X-100 method. This problem was overcome by an additional lysis step using proteinase K, but at a lower concentration than was used in the original method.

The other modification to PFGE was to grow *B. cepacia* to log phase which requires incubation for three hours. This resulted in a faster technique with results obtained a day earlier than with the previous method. However, this advantage is lost when fingerprinting isolates of the 'epidemic' strain of *B. cepacia* as they require an additional overnight incubation step.

When each of the criteria necessary for a good typing system was considered independently, PFGE was successful in fulfilling the majority of the requirements set out by Hawkey (1989). PFGE has the ability to type the majority of strains from most genera of microorganisms. However, different lysing procedures are required to enable extraction of chromosomal DNA from different microorganisms and selection of an appropriate restriction enzyme is necessary to cleave the varying nucleic acid sequences of chromosomal DNA encountered. Digestion of chromosomal DNA results in a range of DNA fragments varying both in the size and number produced. Initial and final pulse times, the most important factors for production of well-resolved banding patterns in PFGE, are dependant on the size range of resultant DNA fragments following cleavage with an appropriate restriction enzyme. Although the majority of isolates can be typed by PFGE, two isolates of *P. aeruginosa* and a single isolate of *B. cepacia*, in this study, were found to be untypeable by this technique. However, it should be stressed that several hundred isolates from each species were typed by PFGE without any problem. Different DNA extraction methods were used in the hope of resolving these problem isolates but without success. Similar findings have been reported by Romling *et al* (1994)

who found that isolates of *P. aeruginosa* from two different patients were untypeable by PFGE. Romling *et al* (1994) speculated the problem was due to a high concentration of endogenous nuclease that could not be inactivated.

PFGE demonstrated good discrimination; indeed it may be argued that it is too discriminatory. However, if the guidelines suggested by Tenover and colleagues (1995) for interpreting DNA restriction patterns generated by PFGE are taken into account, isolates which are closely related can demonstrate up to a three band difference. Furthermore, isolates showing up to a six band difference may be considered related when an epidemiological linkage can be shown.

PFGE showed good reproducibility and enabled comparison of profiles from isolates within the same gel and between different gels. Thus PFGE compares favourably with other genomic typing systems where comparison of isolates between different gels is not reliable - a disadvantage when large numbers of isolates are being investigated.

The only criteria on which PFGE fails to meet the requirements for an ideal typing system for *B. cepacia* is that the technique is technically demanding, time consuming and uses specialised, expensive equipment. However, the majority of the other genomic typing systems also fall into the same category, although some methods are less time consuming to perform and results can be obtained faster than is possible with PFGE.

8.11.2 Comparison of two genomic typing systems

Comparison of the two genomic typing systems PFGE and PCR-ribotyping showed that PFGE is more discriminating and produces more reproducible results. Although PCR-ribotyping demonstrated good reproducibilty of isolates within the same gel, as was observed by repeated isolates of the same strain of *B. cepacia*, there were considerable variations from gel-to-gel. One of the main disadvantages of PCR-ribotyping is that only a few bands are produced; making it difficult, in some cases, to make judgements on the relationship between isolates. Comparing profiles produced by PFGE and PCR-ribotyping, it appeared that any slight variation in banding patterns from PCR-ribotyping such as the intensity or thickness was sufficient to indicate that an isolate was different. However, PCR-ribotyping has the advantage that it is a quicker procedure to perform and results from the PCR amplification and subsequent separation by electrophoresis were achieved within a few hours. The DNA extraction procedure is, however, more time consuming taking a day to complete. Results from PCR-ribotyping can be achieved within 48 hours compared to 3 - 4 days by PFGE.

Although somewhat discredited as a 'phenotypic system', bacteriocin typing was found to be reliable as a step in the identification of *B. cepacia* ET12 and other 'epidemic' strains of *B. cepacia*. Bacteriocin typing can also distinguish other *B. cepacia* strains. However, it was necessary to use the genomic systems to divide isolates of *B. cepacia* within the common S22/P0 group. Nevertheless, in large epidemiological studies, bacteriocin typing is a useful screening procedure.

8.11.3 'Epidemic' strain of *B. cepacia*

The 'epidemic' strain of *B. cepacia*, known as the intercontinental or ET lineage, has been responsible for colonising 15 CF patients in Edinburgh since 1989. All patients attended the adult CF clinic with the exception of a 21-year-old female who continued to attend the paediatric clinic. As previously indicated, there was compelling epidemiological evidence that social contact outwith the hospital was responsible for transmission of this strain in all but one patient. For example, the patient, who attended the paediatric clinic appeared to have acquired the 'epidemic' strain from her CF boyfriend who had acquired the strain some months earlier. Surveillance of ET12 isolates by PFGE showed that there was very little detectable change in ET12 isolates as it transferred from patient-to-patient. The most recent patient to acquire *B. cepacia* ET12 was colonised in 1997; comparison of the PFGE profile of this isolate with that of the index isolate showed only a four band difference in the banding patterns using restriction enzyme *Xba*I and only a two band difference with restriction enzyme *Spe*I.

Longitudinal studies of the Edinburgh index case who acquired ET12 in August 1989 showed only a few minor variations occuring in the PFGE profiles in subsequent years. Using restriction enzyme *Xba*I, variations were observed in the most recent isolate of *B. cepacia* from this patient, taken nine years after the initial isolate; however, the use of another restriction enzyme *Spe*I showed no difference in this isolate when compared to the original.

8.11.4 Genomovars

It appears that the majority of CF isolates of *B. cepacia* of clinical importance belong to genomovar III; these include isolates associated with epidemic spread (Govan *et al*, 1996; LiPuma, 1998) and those responsible for 'cepacia syndrome' within the CF community. However, as demonstrated by the Glasgow 'epidemic' strain which belongs to genomovar II (Vandamme *et al*, 1997), strains of *B. cepacia* demonstrating transmissibility between CF patients are not exclusively found in genomovar III. Although isolates of *B. cepacia* belonging to other genomovar groups have not been associated with patient-to patient transmission it cannot be assumed that these strains are not transmissible. It may be that these strains have not had the opportunity to spread among CF or non-CF patients.

It was initially thought that the cable-like pili encoded by the *cbl*A gene (Sun *et al*, 1995) and/or a novel DNA marker, the *B. cepacia* epidemic strain marker (BCESM) (Mathenthiralingam *et al*, 1997) were associated with transmissiblity as is the case in the ET12 lineage (Govan *et al*, 1996). Further research has shown that not all transmissible strains carry these markers; the 'epidemic' strain of *B. cepacia* appears to be unique in possessing both the *cbl*A and the BCESM markers. Further research work is necessary to identify other transmission factors within the *B. cepacia* complex and to identify the key host/pathogen relationship responsible for virulence. Meanwhile, all members of *B. cepacia* complex must be regarded as having the potential to spread from patient-to-patient and all *B. cepacia*-positive patients segregated from those not colonised with *B. cepacia*.

8.11.5 How far should segregation be practised?

Recent evidence has shown that a number of *B. cepacia*-positive patients colonised with non-epidemic strains of *B. cepacia* have required the ET12 lineage. This worrying development most likely occured through contact at CF clinics. As a result, CF centres are now faced with the logistical and social problem of segregating *B. cepacia*-postive patients colonised with the ET12 lineage of *B. cepacia* from those colonised with other strains of *B. cepacia*.

8.11.6 Are environmental and clinical isolates of *B. cepacia* different?

B. cepacia has attracted considerable interest from the agricultural industry as a bacterial biopesticide and bioremediator to replace the present rather toxic agents. Not unexpectedly, this development is a cause of considerable concern for CF patients and their carers. Phenotypic and genomic studies on clinical and environmental isolates of *B. cepacia* showed that isolates from each source belong to a closely-related taxonomic cluster comprising of five genomovars. Isolates of *B. cepacia* cultured from both clinical and environmental samples are found in all genomovars, although few environmental isolates appear to belong to genomovar III.

This study provided the first unequivocal evidence of an isolate of *B. cepacia* recovered from the sputum culture of a CF patient showing similar properties, both phenotypic and genomic, to that of a phytopathogenic type strain of *B. cepacia* ATCC 25416, which was isolated from an onion. Although this clinical isolate was only detected on a single occasion from the patient's sputum, it nevertheless suggests

that there is the potential for environmental strains to colonise CF patients, albeit transiently.

CHAPTER 9

STENOTROPHOMONAS MALTOPHILIA

9.1 TAXONOMY AND NOMENCLATURE

Stenotrophomonas maltophilia, first identified in 1943 following culture from human pleural fluid, was initially called *Bacterium bookerii*. This organism was then classified as *Pseudomonas maltophilia* in 1961 by Hugh & Ryschenkow but was not included in the Approved List of Bacterial Names (Skerman *et al,* 1980) until the name was revived by Hugh in 1981.

In the early 1980's, the genus *Pseudomonas* consisted of five rRNA homology groups of which *P. maltophilia* designated to groupV. However, genotypic and phenotypic data suggested that this organism was more closely related to the genus *Xanthomonas* than to the *Pseudomonas* group; hence it was proposed to transfer the species *P. maltophilia* to *Xanthomonas maltophilia* (Swings *et al*, 1983). This transfer was not universally accepted as subsequent examination of the properties of the two genera showed clear differences between *P. maltophilia* and the genus *Xanthomonas*. Palleroni & Bradbury (1993) proposed a new genus, *Stenotrophomonas* (Gr. adj. *stenus*, narrow; Gr. n. *trophus*, one who feeds; Gr. n. *monas*, a unit, monad; M.L. fem. n. *Stenotrophomonas*, a unit feeding on few substances) which includes the single species *S. maltophilia*.

9.2 GENERAL CHARACTERISTICS

S. maltophilia is an aerobic, non-fermentative Gram-negative bacillus and is motile with varying numbers of flagella attached at one or both poles of the cell. It possesses distinct biochemical characteristics, including a positive reaction for lysine decarboxylase, aesculin hydrolysis, gelatin hydrolysis, DNase, acidity with maltose and glucose oxidatively, and a weak or negative cytochrome oxidase result.
S. maltophilia normally produces a range of extracellular enzymes including elastase, esterase, protease, hyaluronidase, lipase and mucinase. Protease and elastase are produced by strains of *S. maltophilia* isolated from leukaemic patients with ecthyma gangrenosum (Bottone *et al*, 1986); the importance of these virulence factors in CF isolates has not been analysed. *S. maltophilia* is unable to use ammonia as a nitrogen source when grown in chemically defined medium (Gilardi, 1969) and methionine is a necessary growth factor (Iizuka & Komagata, 1964). Like many of the pseudomonas species, *S. maltophilia* has the ability to grow at 42° C (Gilardi, 1969).

Natural reservoirs of *S. maltophilia* include raw food, rabbit faeces, plants and fresh water (Hugh & Ryschenkow, 1961); *S. maltophilia* is also found in sinks, drains, respirators, and disinfectant solutions in the hospital environment (Moody & Young, 1975).

S. maltophilia exhibits considerable inherent resistance to most antibiotics, including aminoglycosides, β-lactams, carbapenems in particular imipenem,and most fluoroquinolones (Sader *et al*, 1994). Antimicrobial therapy is generally ineffective

for the treatment of *S. maltophilia*. As is the case for *B. cepacia*, there is growing concern to prevent colonisation with such inherently resistant opportunistic pathogens.

9.3 PATHOGENICITY

S. maltophilia was originally considered to be of low pathogenicity. Recently, however, it has emerged as an important nosocomial pathogen, particularly in immuno-compromised patients in critical care units (Maningo & Watanakunakorn, 1995), or patients with haematological malignancies or solid tumours (Fujita *et al*, 1996; Khardori *et al*, 1990), debilitating illness, indwelling catheters or undergoing surgical procedures (Bingen *et al*, 1991; Marshall *et al*, 1989). In these patients, *S. maltophilia* is capable of causing a wide range of infections including respiratory, urinary and eye infections, bacteraemic and postoperative wound infections. Clinically significant infection with *S. maltophilia* is uncommon among healthy individuals but a case of pneumonia has been reported in a non immuno-compromised patient undergoing treatment for bronchiectasis. Interestingly, this is the first reported case of infection due to a mucoid phenotype of *S. maltophilia* (Irifine *et al*, 1994). No mucoid strains of *S. maltophilia* have been reported in CF patients which is surprising considering mucoid strains of *B. cepacia* have been observed occasionally in these patients and that the mucoid phenotype of *P. aeruginosa* is so characteristic of CF pulmonary infection.

Risk factors thought to be associated with contributing to *S. maltophilia* infection include prolonged hospitalisation and mechanical ventilation, prior antimicrobial therapy with broad-spectrum antibiotics, in particular imipenem (Elting *et al*, 1990), haematological malignancies (Fujita *et al*, 1996) and serious underlying illnesses, especially of the respiratory tract (Laing *et al*, 1995). Possible reservoirs for *S. maltophilia*, associated with infection may be the respiratory tract (Khardori *et al*, 1990; Morrison *et al*, 1986), gastrointestinal tract (Elting *et al*, 1990) or natural environments (Talon *et al*, 1994).

9.4 *S. MALTOPHILIA* IN CF PATIENTS

The first reported isolate of *S. maltophilia*, from the sputum of a CF patient was in 1975 in Denmark (Frederiksen *et al*, 1995). From the early 1980s, an increased incidence in the isolation of *S. maltophilia* from sputum culture of CF patients has been reported leading to a prevalence ranging from 1.9% in USA, 11-15% in UK to 30.7% in Spain (Gladman *et al*, 1992; Ballestero *et al*, 1995; Denton, 1997). This increase in prevalence may be partly explained by an increased awareness of the organism, the use of selective medium and more aggresive use of antibiotics (Denton, 1997). As evidence, introduction of selective medium in one UK CF centre resulted in an increase in the isolation rate from 13% to 19% (Denton *et al*, 1996).

The use of potent anti-pseudomonal antibiotics, in particular tobramycin, and a more aggresive antimicrobial therapy, is thought to be a contributing factor in the acquisition of *S. maltophilia* in CF patients (Bauernfeind *et al*, 1992). However, the

emergence of *S. maltophilia* during a double-blind, placebo-controlled trial of inhaled tobramycin in 520 CF patients in USA was higher in the placebo group than in the tobramycin group (Ramsey *et al*, 1999). In Leeds, a large number of CF patients, culture positive for *S. maltophilia*, were treated with ciprofloxacin in the year prior to initial isolation (Denton *et al*, 1996). Although many strains of *S. maltophilia* are sensitive to ciprofloxacin, they rapidly develop resistance during therapy (Lesco-Bornet *et al*, 1992). Eradicating *P. aeruginosa* from the respiratory tract also appears to increase the risk of *S. maltophilia* colonisation. Imipenem has been shown to be a risk factor for *S. maltophilia* in immuno-compromised patients but there is no evidence of CF patients, treated with imipenem, acquiring *S. maltophilia* (Spencer, 1995).

The clinical significance of *S. maltophilia* cultured from CF respiratory secretions is unclear and until recently, colonisation has not been associated with poor prognosis. However, there is a degree of controversy as to whether *S. maltophilia* plays a pathogenic role in CF patients. The Danish CF centre has demonstrated a host immune response to this organism suggesting, that in some patients, it may be invasive and may cause harm (Frederiksen, 1994). Specific antibodies against *S. maltophila* have not been demonstrated (Karpati *et al*, 1994). Colonisation of the lower airways with *S. maltophilia* shows a similar pattern to *P. aeruginosa* with initially intermittent isolation followed by chronic colonisation. Similarily, *S. maltophilia* is rarely eliminated and when this occurs it is generally only for short periods of time (Karpati *et al*, 1994).

Extensive studies by Karpati *et al* (1994) showed that no clinical deterioration was observed in CF patients colonised with *S. maltophilia* for up to two years. However, elevated WBC count and/or ESR values were observed when compared to matched patients colonised with *P. aeruginosa* alone. Patients colonised with *S. maltophilia* for a period greater than two years showed a gradual deterioration in lung function and it was concluded that *S. maltophilia* played a pathogenic role in these CF patients. The investigation did not identify any predisposing factors contributing to colonisation in CF patients such as previous colonisation with other bacteria, antimicrobial therapy or pulmonary function. Gladman *et al* (1992), concluded that *S. maltophilia* was not directly responsible for mortality.

9.5 EPIDEMIOLOGY

The epidemiology of *S. maltophilia* is unclear; in particular, whether acquisition occurs through person-to-person transmission or from environmental sources. However, a number of typing techniques for *S. maltophilia* have been developed which should facilitate epidemiological investigations. Various phenotypic characteristics have been used to compare isolates of *S. maltophilia*, such as biochemical tests and antibiograms, but no method has been used extensively and those available are of little use for epidemiological studies. Serological typing has been applied to *S. maltophilia* and is based on heat stable somatic O antigens detected by agglutination; serotyping , however, shows poor discrimination and the antisera are not widely available (Schable *et al*, 1989). Other phenotypic typing techniques which have been applied to investigation of nosocomial outbreaks of

S. maltophilia include multilocus enzyme electrophoresis (Schable *et al*, 1991) and pyrolysis mass spectrometry (Orr *et al*, 1991); both systems require specific and rather expensive, specialised equipment. In general, phenotypic typing has proved inadequate in determining the route of transmission of *S. maltophilia* and it would appear that the more discriminating genomic typing systems are necessary.

Recently, several genomic typing systems have been developed for the fingerprinting of *S. maltophilia*; these include ribotyping, PFGE, enterobacterial repetitive intergenic consensus ERIC-PCR and two PCR typing methods, arbitrarily primed (AP-PCR) or random amplified polymorphic DNA (RAPD).

In a Danish CF centre, Frederiksen *et al* (1995) used ribotyping to show two small clusters of CF patients with the same strain of *S. maltophilia*, one group of five patients and the other group with only two patients. All five patients had attended the CF clinic on the same day but otherwise had no other contact with each other; whereas there was no evidence of cross-infection between the two patients in the other smaller cluster. Small incidents of cross-infection due to *S. maltophilia* in non-CF patients have also been detected using ribotyping and restriction fragment length polymorphism, with both systems demonstrating excellent correlation. (Bingen *et al*, 1991). A later study, in Copenhagen, on nosocomial isolates of *S. maltophilia* revealed a small group of four patients harbouring the same ribotype; however, no epidemiological relationship was determined (Gerner-Smidt *et al*, 1995).

A number of investigators have used PFGE for typing strains of *S. maltophilia* and no evidence of cross-infection among CF patients has been reported (Talon *et al*, 1994, VanCouwenberghe *et al*,1995). However, Marty (1997) has shown environmental isolates with the same genotype as those responsible for causing nosocomial infections. PFGE also demonstrated the presence of the same strain of *S. maltophilia* over a 15 month period, in a CF patient, with no change in bacterial characteristics apart from an increase in resistance to antibiotics (Wust *et al*, 1995).

AP-PCR has been shown to be marginally less discriminating than PFGE for typing nosocomial isolates of *S. maltophilia* (VanCouwenberghe *et al*, 1995). Investigations using two PCR techniques, RAPD and ERIC, have demonstrated that strains of *S. maltophilia* from some CF patients have closely related profiles (Chatelut *et al*, 1995); in contrast, Denton *et al* (1996) found no evidence of cross-infection among CF patients using ERIC-PCR.

9.6 AIMS

Since the incidence of *S. maltophilia* recovered from sputum cultures of CF patients has greatly increased in recent years, the aim of this study was to determine the prevalence of *S. maltophilia* among CF patients in Edinburgh and to investigate whether the increased incidence of *S. maltophilia* was associated with person-to-person transmission.

9.7 RESULTS

At the time of this study, there were 155 CF patients in Edinburgh, comprising of 79 paediatric and 76 adult patients. At regularly three monthly visits all sputum and throat swabs were cultured for *S. maltophilia* using selective medium; 121 isolates were obtained from 37 different CF patients. The rate of incidence of *S. maltophilia* in the 37 patients, comprising of 22 children and 15 adults, was 28% and 20%, respectively.

Multiple isolates of *S. maltophilia* were typed by PFGE, using restriction enzyme *Xba*I, to determine the diversity of the PFGE profiles prior to any subsequent epidemiological studies of this organism. *Xba*I digestion of DNA extracted from *S. maltophilia* produced 18 -25 fragments with a size range of <48.5 - 582kb. The range of PFGE profiles obtained from nine different CF isolates of *S. maltophilia* are shown in Fig 9.1 and demonstrate the genetic diversity of this organism.

To determine the clonal relationship, if any, among the Edinburgh isolates of *S. maltophilia* each isolate was genomically fingerprinted by PFGE, using restriction enzme *Xba*I. The results demonstrated that the majority of patients were colonised with their own unique strain(s) of *S. maltophilia*, with the exception of three small clusters of paediatric patients. These clusters consisted of four patients in group I and two patients in each of the groups II and III. Within group I, there are two sets of two patients with isolates of *S. maltophilia* which produced identical PFGE profiles.

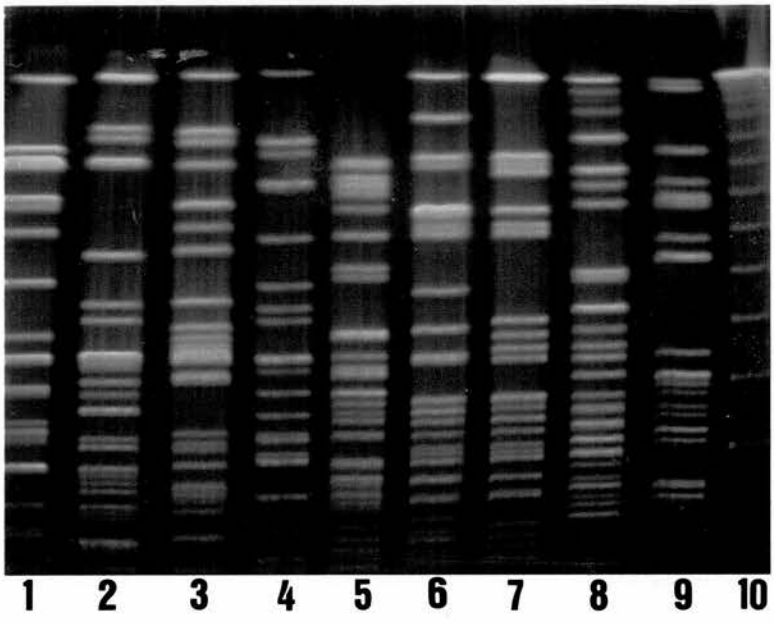


FIG. 9.1. *Xba*I digestion of DNA from nine different CF isolates of *S. maltophilia* showing a range of PFGE profiles. Lanes 1 to 9, C1954, C1950, C1936, C1932, C 1953, C 1970, C1972, C1969, C1914; lane 10, lambda concatemers (size range 48.5kb to 582kb, in increments of 48.5kb).

However, comparison of the profiles produced by each set of patients revealed a six band difference, indicating that the four isolates within group I may be related. The PFGE profiles produced by the two isolates in groups II and III were found to be identical in each case (Fig 9.2).

Interestingly, two patients in group III, isolates C1932 and C1955, acquired *S. maltophilia* within a month of each other and in both cases the isolates produced a melanin-like pigment, which is an unusual characteristic for *S. maltophilia*. Sharing of this unusual phenotype suggests that patient-to-patient transmission had ocurred. To date, both patients continued to harbour the same strain but there has been no evidence of transfer to any other CF patient. *S. maltophilia* was isolated from only one pair of siblings and surprisingly, their isolates appeared to be unrelated. Interestingly, all isolates from the adult CF patients produced unique genotypic profiles indicating that the patients are colonised with unrelated strains of *S. maltophilia*.

Multiple isolates of *S. maltophilia* were obtained from 13 of the 37 CF patients, who were colonised for more than six months, to investigate whether the patients remained colonised with the same strain of *S. maltophilia.* PFGE demonstrated that ten of the 13 patients harboured the same strain for a period of time ranging from six months to three years. The remaining three patients were found to be colonised by different strains over the same period with one patient having three different strains within a year.

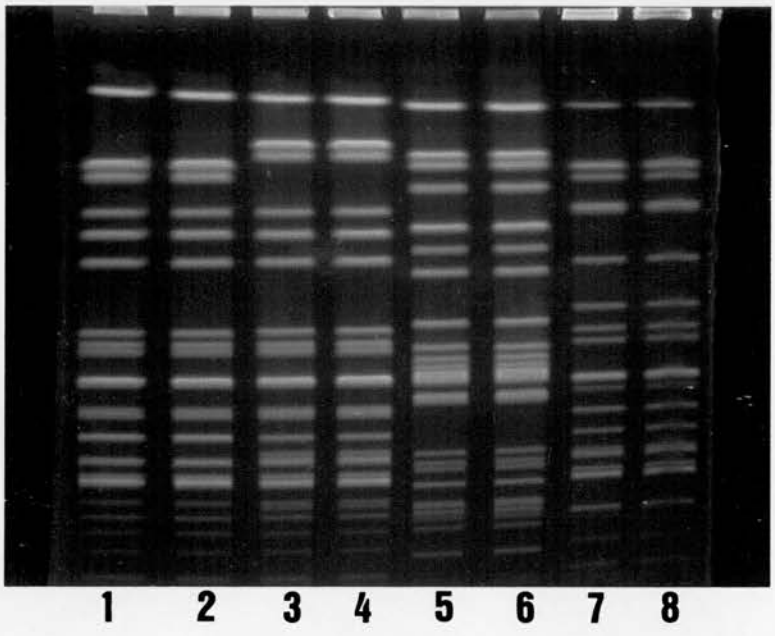


FIG. 9.2. *Xba*I digestion of isolates of *S. maltophilia* demonstrating three clusters of CF patients colonised with similar strains. Lanes 1 to 4, group I - C1912, C1933, C1981, C1980; lanes 5 & 6, group II - C1934, C1936; lanes 7 & 8, group III - C1932 & C1955.

Further investigations were carried out on eight isolates of *S. maltophilia* from one patient cultured over a period of three years. The PFGE profiles using restriction enzyme *Xba*I (Fig 9.3), showed that six of the isolates were related although when compared to the original isolate, C1925, up to a four band difference was observed. Similar differences, up to a four band difference, were observed when a second restriction enzyme *Spe*I was used to digest the DNA from the same six isolates (Fig 9.4). According to the criteria set out by Tenover *et al* (1996), isolates with up to a three band difference in PFGE profile are closely related and regarded as the same strain while isolates showing up to a six band difference are considered as possibly related. In this patient, the most recent isolates, C1971 and C1981, produce identical profiles indicating that they are the same strain but differ from the original.

The six isolates (C1925, C1915, C1939, C1931, C1941, C1973) were further examined by biochemical tests and antimicrobial susceptibility tests to determine whether the differing PFGE banding patterns were associated with any distinguishing phenotypical markers. Further investigations included colonial morphology and assay for DNase, lysine and ornithine decarboxylase, protease, and elastase.

Results showed that there was no difference between the isolates in colonial morphology, elastase was not produced by any isolate and all isolates gave positive reactions for DNase, LDC, ODC, and protease.

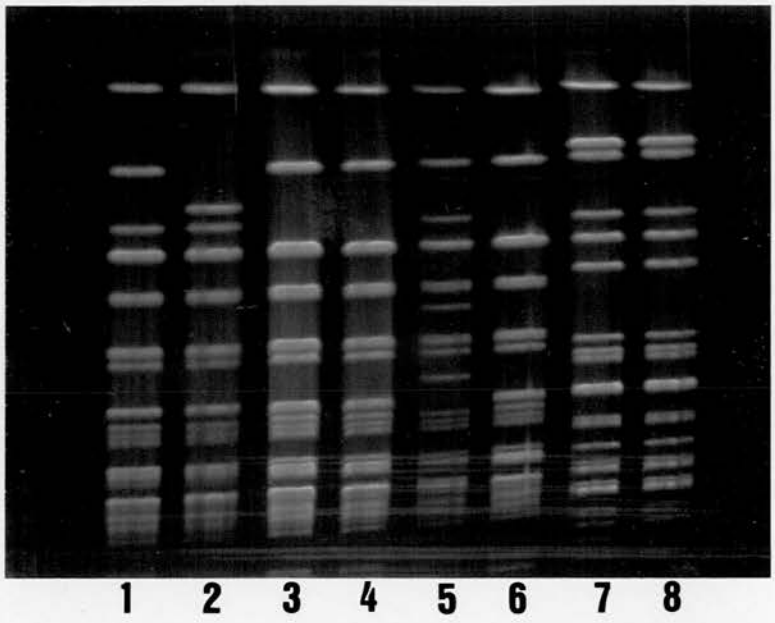


FIG. 9.3. *Xba*I digestion of eight isolates of *S. maltophilia* from the same CF patient. Lanes 1 to 8, C1925, C1915, C1939, C1931, C1941, C1973, C1971, and C1981.

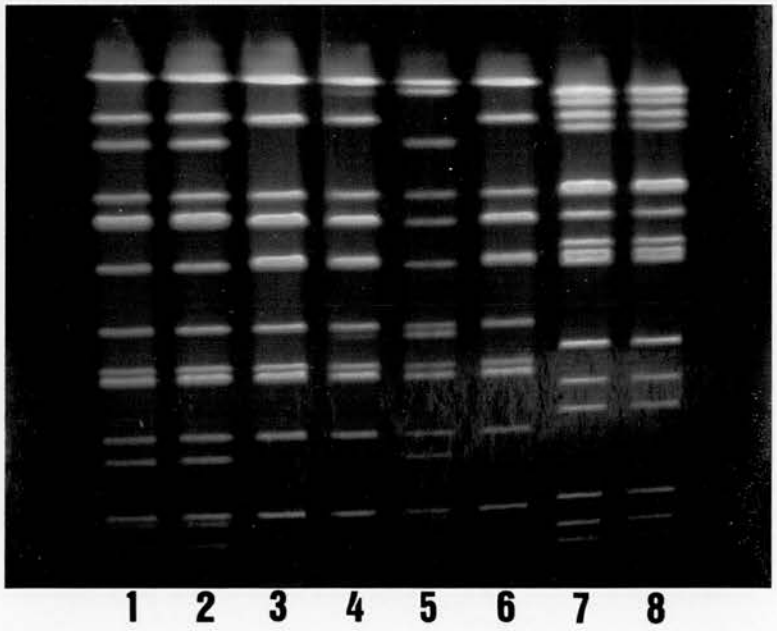


FIG. 9.4. *Spe*I digestion of the same eight isolates of *S. maltophilia.* Lanes 1 to 8, as above.

Examination of the API 20NE profiles also showed identical patterns, with the exception of C1931 which was ONPG negative. These results suggest that the PFGE banding differences were not associated with any difference in biochemical or sugar assimulation tests.

It was then decided to investigate the antibiogram patterns of the six isolates to the following antibiotics: ceftazidime, ciprofloxacin, gentamicin, tobramycin, imipenem, azlocillin, colomycin, cotrimoxazole and aztreonam. The only difference in antimicrobial susceptibility patterns were found with imipenem, which revealed that strains C1931, C1941 and C1973, which gave identical PFGE patterns, were all sensitive to imipenem whereas the other isolates were resistant.

The minimum inhibitory concentration (MIC) of imipenem to isolates C1931, C1941 and C1973 was 2 μg/ml compared to the other isolates which had an MIC >32 μg/ml. Could this account for a change in the banding pattern? To answer this question, an imipenem resistant mutant of isolate C1941 was made (MIC of 35 μg/ml) and then fingerprinted by PFGE to determine whether there was a difference in the profile pattern compared to the original strain. The results showed that both the sensitive and the resistant strains produced identical PFGE profiles indicating that resistance, at least in this mutant, does not necessarily produce a change in PFGE profile.

9.8 DISCUSSION

In recent years, a number of epidemiological studies have been carried out to determine the relatedness of strains of *S. maltophilia* causing nosocomial infections in immuno-compromised patients and patients with CF. To date, patients tend to be infected or colonised with their own unique strain of *S. maltophilia* and there is little or no evidence of cross-infection among patients. However, Laing *et al* (1995) observed a cluster of six patients in an intensive care unit, over a seven month period, with isolates producing identical DNA profiles. These findings suggest that either cross-infection had occurred between the patients or that the patients had been infected from a common source which was not determined.

The results from this present study on isolates of *S. maltophilia*, from CF patients, revealed that the majority of patients are generally colonised with their own strain of *S. maltophilia*. These findings are similar to those encountered with other CF pathogens, including *P. aeruginosa* and *B. cepacia* (apart from patients colonised with 'epidemic' strains of *B. cepacia*), namely that patients tend to be colonised with their own strain of the particular organism. However, the presence of the three small clusters of paediatric CF patients with each patient within the group being colonised with *S. maltophilia* producing the same PFGE pattern provides evidence of possible patient-to-patient transmission. Similar findings were observed in Denmark using ribotyping. Again, only small clusters of patients with the same strain were observed (Frederiksen, 1994). In contrast, other studies have found no evidence of cross-infection in CF patients, using ERIC-PCR and PFGE respectively (Denton *et al*,

1996 and VuThien *et al*,1996). As cross-infection appears to account for only a small number of CF patients acquiring *S. maltophilia*, other contributing factors must also be responsible for the increased incidence of *S. maltophilia* in CF patients.

The risks factors associated with nosocomial infections caused by *S. maltophilia* have been reported as long term hospitalisation, prior antimicrobial treatment, and debilitating illnesses. In recent years, however, management of CF pulmonary disease has changed with CF patients spending less time in hospital due to the increased administration of intravenous antibiotics at home. Thus, long term hospitalisation does not appear to be a likely cause for the increased incidence in isolation of *S. maltophilia* from sputum cultures from CF patients.

Further analysis of the 37 CF patients in this study colonised with *S. maltophilia* revealed that 18 (49%) were colonised with *P. aeruginosa* prior to acquisition of *S. maltophilia*. Interestingly, no patients were previously colonised with *B. cepacia*. Bauernfeind *et al* (1992) suggested that tobramycin may act as a contributing factor in the acquisition of *S. maltophilia*. This explanation appears feasible as patients colonised with *P. aeruginosa* will almost certainly have been treated with widely-used anti-pseudomonal agents. However, some patients who are colonised with *S. maltophilia* have never had *P. aeruginosa* cultured from their sputum and subsequently have not been exposed to anti-pseudomonal antibiotics. In such cases, antimicrobial therapy is not a predisposing factor for the acquisition of *S. maltophilia* and there are obviously other factors involved. However, there must be large

numbers of CF patients colonised with *P. aeruginosa* who have received treatment with tobramycin and have not acquired *S. maltophilia*, even transiently.

The observation, in this study, that sensitive and resistant strains of *S. maltophilia* produced identical banding patterns by PFGE has also been confirmed by Wust *et al* (1995) who also found that strains with different antibiograms showed no difference in PFGE profile. It is concluded that variation in phenotypic properties, at least in some forms of antibiotic resistance, appear not to have any affect on PFGE banding patterns.

CHAPTER 10

GENERAL CONCLUSIONS

10.1 PFGE

At present, genomic fingerprinting by PFGE is considered by many microbiologists to be the 'gold standard' for molecular typing systems. In most bacterial species, PFGE provides good discrimination, reproducibility and stability i.e. the criteria required for a reliable typing system. The only disadvantages encountered with PFGE during the preliminary studies for this thesis was that it was time-consuming and required considerable technical expertise and expensive equipment. However, as discriminatory power and reproducibility are the most important aspects of a typing system, PFGE can almost be regarded as ideal. Thus for the purpose of this thesis, PFGE was chosen as the typing system for epidemiological investigation of the four main CF pathogens, *Staph. aureus*, *H. influenzae*, *P. aeruginosa*, *B. cepacia*, and the potential pathogen *S. maltophilia*.

During the thesis, development of a new procedure for extraction of chromosomal DNA from isolates of both *B. cepacia* and *P. aeruginosa*, and subsequent separation of the digested DNA by PFGE, resulted in improved PFGE banding patterns. Not only are the banding patterns more clearly defined and more easily interpreted but the new extraction technique produces consistently good results.

PFGE banding patterns for different isolates can be compared by visual observation. However, difficulties can arise when attempts are made to compare the patterns of new isolates with a large number of previous isolates on different gels. Various computer-assisted systems for analysing DNA fingerprinting gels have been developed in an attempt to overcome this problem. One of the disadvantages of these systems is that at least three molecular size markers have to be run on each gel to allow comparison of banding patterns within and between different gels. As there are only ten lanes in the majority of PFGE gels, the inclusion of markers restricts the number of samples that can be run on a gel at the same time. This is a particular disadvantage if there is a need to investigate a large number of strains.

Some of the computerised systems do not produce a high correlation value even when banding patterns are visually identical. A study of two systems, GelCompar and DENDRON, revealed that they were good for identifying closely related strains but showed considerable variation in less closely related strains (Seward *et al*, 1997). These authors also showed a lack of confidence in these two systems by suggesting that results from the computerised systems should always be checked visually as discrepancies can occur.

Comparison of two PCR-based genomic typing systems, PCR-ribotyping and RAPD, with PFGE showed the PCR systems to be less discriminating for isolates of *B. cepacia* examined in this thesis. In the initial studies, considerable difficulty was encountered with the RAPD technique and even after some of the problems were

overcome the technique lacked reproducibility. Consequently, RAPD was not considered for any of the epidemiological investigations in this thesis. PCR-ribotyping was easy to perform and reproducible but only a few bands are produced and interpretation of results can be difficult following electrophoresis of the PCR product. Differences in PFGE profiles, often involving up to 30 bands, provided convincing evidence to distinguish individual strains whereas the banding patterns produced by PCR-ribotyping did not provide good evidence to judge clonal relationships between isolates. However, PCR-based typing techniques have the advantage that they are quicker and easier to perform and require less expensive equipment than PFGE.

10.2 *STAPHYLOCOCCUS AUREUS*

Staph. aureus is commonly found in the anterior nares of many healthy individuals without causing adverse affects. However, considerable differences have been observed in the nasal carriage rates of *Staph. aureus*, even in healthy individuals, ranging from 25% in this study to 37.2% in a study by Kluytmans and colleagues in 1997.

In this study, the nasal carriage of *Staph. aureus* was found to be unexpectedly high in the adult CF patients. This contrasted with carriage in the paediatric group which was virtually the same as in the normal population. There is no obvious explanation to account for this variation in nasal carriage rates of *Staph. aureus* between the

paediatric and adult CF patients or to explain why the incidence is so high in the adult patients.

Isolates of *Staph. aureus* cultured from the anterior nares have been shown to cause endogenous infections in non-CF patients (Kluytmans *et al*, 1997); it has always been assumed that a similar endogenous route applied to CF patients. However, this thesis provided striking evidence that in 76% of CF patients isolates of *Staph. aureus* recovered from nasal swabs and sputum were clonaly unrelated. Furthermore, although transient strains were detected the presence of a strain of *Staph. aureus* in the sputum appeared to prevent the acquisition of another strain. Data from these investigations also showed a patient could harbour the same strain of *Staph. aureus* in their nose for at least two years (the duration of the investigation).

Studies on the nasal flora of the 16 CF patients who participated in the gene therapy trials revealed that the invasive treatment, involving the application of a liposome/gene complex to the anterior nares, did not appear to have any affect on the nasal flora. Although more than one isolate of *Staph. aureus* was recovered from nasal swabs from a few patients there was no evidence that the treatment was responsible.

10.3 *HAEMOPHILUS INFLUENZAE*

Following the successful development of a method for the extraction of chromosomal DNA from isolates of *H. influenzae*, and digestion with an appropriate

restriction enzyme, the subsequent DNA fragments were separated by PFGE. This achievement enabled a comparison between PFGE and the traditional phenotypic typing system for non-capsulate *H. influenzae*, namely biotyping. Although as expected, PFGE was found to be more discriminatory than biotyping, a reasonable degree of correlation was observed. For example, in no instance did isolates of *H. influenzae* producing the same PFGE pattern belong to a different biotype.

Interestingly, a difference in the biotype distribution of *H. influenzae* between CF and non-CF patients was observed with the majority of isolates of *H. influenzae* from the non-CF patients belonging to biotype II. In contrast, isolates of *H. influenzae* recovered from throat swabs and sputum from CF patients did not demonstrate a predominant biotype. The majority of isolates belonged to biotypes I, II or III, with similar numbers in each of the biotypes. When CF sputum isolates of *H. influenzae* were analysed separately, a high proportion of the isolates belonged to biotype I. The significance of *H. influenzae* in respiratory secretions in CF patients is a constant subject of debate, in particular, whether *H. influenzae* is acting as a pathogen or merely a respiratory commensal. However, large numbers of *H. influenzae* present in sputum culture have been associated with pulmonary exacerbations and high levels of pro-inflammatory cytokines suggesting that the organism is a potential pathogen. Taking this into account and the fact that the majority of isolates recovered from sputum samples were biotype I, this may indicate that biotype I organisms may play a role in producing exacerbations in CF patients. However, the number of isolates of *H. influenzae*, recovered from sputum culture and analysed in this study, is probably

too small to make such an assumption. There was also no evidence to suggest that particular strains of *H. influenzae* are responsible for exacerbations in CF patients.

It is a well known dogma of CF microbiology that *Staph. aureus* and *H. influenzae* tend to cause infection in infants and young patients whereas, with the usual exceptions, *P. aeruginosa* generally appears during adolescence. Thus, the recovery rate of *H. influenzae* is significantly lower in adult CF patients compared to the paediatric group. An interesting observation made during this study was that *P. aeruginosa* can inhibit the growth of *H. influenzae* providing at least one explaination as to why the latter organism appears to be less prevalent in CF adults.

10.4 *PSEUDOMONAS AERUGINOSA*

P. aeruginosa is arguably one of the most important pathogens in CF microbiology. Thus numerous epidemiological studies of this classic bacterial opportunist have been undertaken employing phenotypic and genomic fingerprinting typing systems. In this study, PFGE has shown that the phenotypic typing system of bacteriocin or pyocin typing (Fyfe *et al*, 1984) lacks discriminatory powers, reproducibility and stability. However, pyocin typing has a valuable role to play as a screening procedure when large numbers of *P. aeruginosa* are involved.

Despite surprisingly little scientific evidence another dogma of CF microbiology has been that the characteristic mucoid colonial form of *P. aeruginosa* recovered from the sputum of CF patients emerges as a variant of the original non-mucoid form

responsible for primary colonisation. This speculation was confirmed using PFGE which demonstrated that both colonial morphotypes produced identical profiles. However, our evidence also indicates that it cannot be assumed that the mucoid morphotype is always the same strain of *P. aeruginosa* as the original non-mucoid isolate. This study, of 12 paired isolates of *P. aeruginosa* (original non-mucoid and first mucoid isolate) cultured from sputum from different CF patients revealed that identical PFGE profiles were produced by both the mucoid and non-mucoid isolates in only half of the patients. The PFGE profiles produced by the paired isolates from the other six patients were different indicating that the mucoid morphotype was a different strain of *P. aeruginosa* from the original non-mucoid isolate. The time interval from the recovery of the first isolate of non-mucoid *P. aeruginosa* until it converted to the mucoid form did not appear to have any relevance as to whether both colonial morphotypes were the same strain. A possible explaination is that the original non-mucoid strain of *P. aeruginosa* was replaced by another strain which subsequently converted to the mucoid morphotype.

Although patients may harbour more than one strain at a particular time several studies have employed phenotypic typing to indicate that CF patients are colonised with a predominant strain of *P. aeruginosa* over long periods. Longitudinal studies of the Edinburgh CF patients, extending over two years, revealed that such strain stability occurred in the majority of patients. However, PFGE and pyocin typing suggested that a large number of the patients harboured more than one strain of *P. aeruginosa* in their sputum and these did not always include the original

colonising strain. As different strains of *P. aeruginosa* may exhibit similar colonial morphology, it is possible that the original strain may have been present in the sputum culture and failed to be selected for typing.

P. aeruginosa is generally not associated with episodes of cross-infection in CF patients with the exception of siblings (Grothues *et al*, 1988), small clusters of patients with the same strain in Denmark (Hoiby & Rosendal, 1980), and the spread of a β-lactam resistant strain in Denmark(Pedersen *et al*, 1986). It should be noted that these investigations were carried out using phenotypic typing systems. In particular, the study reported by Pedersen *et al* (1986), and often used as evidence of cross-infection, employed serotyping and phage typing. In this particular study the data lacks conviction since the 'epidemic strain' was polyagglutinating and possessed no clear serotype.

During this thesis, PFGE and flagellin gene polymorphisms (Cheng *et al*, 1996) were used to provide unequivocal scientific evidence of *P. aeruginosa* cross-infection in 55 of 92 *P. aeruginosa* colonised CF patients treated in the Liverpool clinic. Further investigations suggested that the β-lactam ceftazidime resistant strain of *P. aeruginosa*, responsible for this outbreak, emerged as a result of monotherapy. However, this hypothesis does not explain how cross-infection occured amongst so many CF patients. Recent evidence from genomic and phenotypic analyses suggests that this strain has spread further and has been acquired by CF patients in Edinburgh, Manchester and Nottingham. Epidemiological investigation indicates that social

contact occurred between the patients involved, however, no evidence of transmission has been observed amongst the Edinburgh patients. Further investigation of this strain is warranted as it may provide a model of a lineage with high transmissibility, similar to the ET12 transatlantic lineage of *B. cepacia*. This interesting and clinically-relevant *P. aeruginosa* may also be a prime candidate for sequencing following the successful sequencing of the classic PAO1 strain.

10.5 *BURKHOLDERIA CEPACIA*

Longitudinal studies involving the first CF patient in Edinburgh to acquire the 'epidemic' or ET12 transatlantic lineage of *B. cepacia* in 1989, showed that the strain varied little over the nine-year period in which the patient has remained colonised. Interestingly, although many environmental and clinical *B. cepacia* have been examined only an occasional CF isolate has shown a mucoid colonial morphotype. No conversion from the non-mucoid was noted and the exopolysaccharide was not alginate (Govan & Tatnell, unpublished data).

Of the 15 Edinburgh CF patients, who have acquired the 'epidemic' strain of *B. cepacia* only slight variation in the PFGE profiles was detected; interestingly, these differences occurred in the most recent patients to acquire this strain. However, none of these variations could be linked to phenotypic changes in the organism. These studies reveal that isolates of *B. cepacia*, certainly the 'epidemic' strain, do not appear to undergo major phenotypic changes even after many years in the lung environment. This is in striking contrast to *P. aeruginosa* which undergoes

considerable phenotypic adaptation (involving colonial morphology and LPS structure) as it adapts to the CF lung.

B. cepacia also differs from *P. aeruginosa* in that certain strains of *B. cepacia* exhibit enhanced transmissibility amongst CF patients. Recently, *B. cepacia* has been further divided into five subgroups or genomovars I - V based on polyphasic analyses of total protein profiles and DNA-DNA and DNA-rRNA hybridisation. Of these genomovars, the first three have fuelled the most interest in research. Genomovar I generally contains non-transmissible environmental isolates. Genomovars II (recently renamed *B. multivorans*) and III contain strains responsible for major outbreaks in CF clinics. Current evidence suggests that strains associated with the most severe clinical outcomes (including cepacia syndrome) in CF populations from various geographic locations belong to genomovar III. The highly transmissible isolate J2315, which represents the transatlantic ET12 lineage belongs to genomovar III. Strains from genomovars IV and V (renamed *B. vietnamiensis*) have occasionally been isolated from CF patients, but their clinical importance is unclear. At present, the group of five closely related genomovars is referred to as the *B. cepacia* complex.

Transmissible strains of *B. cepacia* are not confined to genomovar III as the Glasgow 'epidemic' strain belongs to genomovar II. There is still not sufficient evidence to say that strains belonging to the other genomovars I, IV, and V are not transmissible. Furthermore, following recent evidence that the ET12 lineage can super infect

B. cepacia positive individuals with fatal consequences, there have been calls for more severe segregation.

Evidence of an environmental/CF link is of major importance in the ongoing debate of *B. cepacia* as a biopesticide and bioremediator. One of the arguments made by microbiologists advocating the use of *B. cepacia* as a biopesticide is that there is no evidence of humans acquiring *B. cepacia* from the environment and hence no 'danger' from increasing the environmental population of *B. cepacia* by commercial exploitation. This argument disregards the fact that *B. cepacia* is not a human or animal commensal and thus must be acquired from the environment. One of the most potentially significant findings of this thesis was evidence to support the human acquisition of *B. cepacia* from the environment. The environmental isolate ATCC 25416 is the original *B. cepacia* type strain isolated from an onion by Burkholder in 1949. During this thesis, ATCC 25416 was found to be similar to a clinical isolate in a number of aspects including onion rot, antibiogram profiles, colonial morphology, identical PFGE profiles and amplified fragment length polymorphism (AFLP) analysis and chromosome size. Although this strain of *B. cepacia* was only isolated from the CF patient on one occasion it nevertheless demonstrates the potential for an environmental isolate to infect a CF patient, albeit for a short period.

10.6 *STENOTROPHOMONAS MALTOPHILIA*

Although *S. maltophilia* is recovered with increased frequency from respiratory secretions of CF patients the microbiological role of this organism still remains

unclear. In this study, evidence of cross-infection was limited and observed in only three small groups of CF patients. This does not explain the increase in incidence of *S. maltophilia* in recent years.

The use of anti-pseudomonal antibiotics, particularly tobramycin and imipenem, have been associated with the emergence of *S. maltophilia* in CF patients. However, in this study only 49% of the Edinburgh patients were colonised with *P. aeruginosa* prior to acquisition of *S. maltophilia* and subsequently 51% of patients had not been exposed to anti-pseudomonal antibiotics. Thus, it would seem that factors other than the use of anti-pseudomonal antibiotics must be involved.

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Spread of β-lactam-resistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic

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Summary

Background *Pseudomonas aeruginosa* colonisation of the airways of patients with cystic fibrosis (CF) is associated with considerable respiratory morbidity. Although segregation of colonised patients from non-colonised patients to prevent cross-infection has been recommended, there is little evidence that such cross-infection is widespread. We observed that a high proportion of children attending our CF clinic were colonised with *P aeruginosa* that was resistant to ceftazidime and other β-lactam antibiotics. We used two genomic fingerprinting techniques to see whether this may have arisen from epidemic spread of a single strain.

Methods The prevalence of *P aeruginosa* colonisation and the antibiotic susceptibility of the organisms was determined from review of laboratory reports in the case-notes of 120 children with CF. Isolates were cultured from the sputum of 65 children colonised with ceftazidime-resistant *P aeruginosa*. Polymorphisms in total bacterial DNA from 92 isolates were analysed with two molecular fingerprinting techniques—pulsed-field gel electrophoresis after restriction enzyme digestion and assessment of flagellin gene polymorphisms by amplification of the whole gene and restriction enzyme digestion.

Results 92 (76·7%) of 120 children were colonised with *P aeruginosa*, and 65 of the 92 harboured isolates that were resistant to ceftazidime. Only three of the 92 children had never been treated with ceftazidime. The results of the two molecular-fingerprinting techniques were concordant and showed that 55 of 65 children harboured the same epidemic strain. This strain was resistant to ceftazidime, azlocillin, and imipenem, and sensitive to tobramycin and ciprofloxacin.

Interpretation This study provides the first molecular evidence of a long-term outbreak of *P aeruginosa* in a CF centre. We suggest that careful surveillance of the prevalence of antibiotic resistance in CF centres should be instituted with measures to prevent cross-infection. We believe that antipseudomonal monotherapy should be considered with caution.

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Introduction

Colonisation with *Pseudomonas aeruginosa* and *Burkholderia cepacia* is associated with progressive lung disease and mortality in patients with cystic fibrosis (CF).[1,2] There is also compelling evidence that epidemic spread of highly transmissible strains of *B cepacia* influences the prevalence of pulmonary colonisation in some centres.[3,4] By contrast, epidemic spread of *P aeruginosa* in unrelated patients, either in CF clinics or through social contacts, is a contentious issue, and there is a need for scientific evidence to support a policy of segregation as practised for patients colonised with *B cepacia*.

Historically, the use of simple phenotypic typing systems suggested that CF siblings tend to harbour the same *P aeruginosa* strain, which indicates the possibility of cross-infection if personal contacts are intimate and frequent. More recently, this form of person-to-person spread has been confirmed by reliable DNA-based genomic typing techniques, including pulsed-field gel electrophoresis.[5,6] Epidemic spread of multidrug-resistant *P aeruginosa* in unrelated patients has also been reported; Pedersen and colleagues[7] showed that the incidence of new cases was reduced by strict isolation of colonised patients. However, their hypothesis that a single strain of *P aeruginosa* was responsible for cross-infection was criticised[8] on the grounds that the study was based on the use of phage-typing and serotyping—phenotypic techniques that are unreliable for characterising the mucoid, polyagglutinable *P aeruginosa* that is characteristic of CF.[9] Nevertheless, institution of cross-infection control measures in this unit interrupted spread of the bacterium. Subsequently, the development and use of genomic typing methods have indicated that small groups of patients within a CF clinic may harbour the same strain, which suggests that cross-infection, or acquisition from a single source, can happen.[6,10]

In contrast to the above reports, most epidemiological studies of patients with CF have not shown spread of *P aeruginosa* in association with attendance at summer camps[11–13], or when sharing hospital rooms,[14] or during regular attendance at a CF clinic over many years.[2] However, there is general agreement[15] that patients with CF attending a CF centre usually harbour individual unrelated strains of *P aeruginosa* over a period of many years, and that, although cross-infection can occur, it is usually restricted to CF siblings or a minority of patients, the precise mode(s) of spread remaining unclear.

In 1995, we became aware that a large number of patients were colonised with a ceftazidime-resistant strain of *P aeruginosa* among the 120 patients who received their CF care in Liverpool but not among the 150 other patients who were regularly reviewed by our CF team at their local hospital. Here, we report the use of two genomic fingerprinting techniques to provide the first unequivocal evidence of an extensive epidemic in unrelated CF patients involving a β-lactam-resistant strain of *P aeruginosa*.

Methods

Patients

Case-notes of 120 patients attending the CF clinic at Alder Hey Children's Hospital in August, 1995, were retrospectively assessed to identify those colonised with *P aeruginosa* and to determine the date of first isolation. Diagnosis of CF was based on two positive sweat tests and confirmed by genetic testing. Antibiotic susceptibility for *P aeruginosa* isolates based on disk-diffusion tests was available from case-notes. According to the treatment policy of the unit from 1986 to 1994, all patients with chronic *P aeruginosa* colonisation (ie, present on three or more occasions) and clinical respiratory deterioration were treated with intravenous ceftazidime for 2 weeks provided that recent isolates were sensitive. If not, alternatives such as ciprofloxacin, aminoglycosides, or colistin were used. Before this study, although patients with *B cepacia* were routinely segregated from others in the clinic, we did not specifically isolate patients with *P aeruginosa*.

Bacterial isolates

In a prospective study, isolates cultured from sputum collected in March, 1995, from a cohort of 65 patients, presumptively identified as having ceftazidime and other β-lactam-resistant *P aeruginosa*, were confirmed as *P aeruginosa* with standard biochemical reactions[16] and the API 20 NE system (BioMerieux, Basingstoke, UK). Antibiotic susceptibilities to antipseudomonal agents were determined initially by the Stokes diffusion technique; subsequently, minimum inhibitory concentrations (MICs) of antipseudomonal agents were assessed with agar dilution methods.[17] Using two independent genomic fingerprinting techniques, we analysed the clonal relationships between the *P aeruginosa* isolates from the 65 patients. Polymorphisms in total bacterial DNA from 92 isolates were analysed with pulsed-field gel electrophoresis (CHEF DRII system, BioRad, Hemel Hempstead, Hertfordshire, UK) following digestion with the endonucleases *Xba*I and *Spe*I. We used the method as previously described for *B cepacia*[18] except that pulses were 2–28 at 200 V for 20 h at 14°C.

In the flagella of *P aeruginosa* the two major types of flagellin (a and b) are differentiated on protein size and antigenicity. We have designed PCR primers based on the conserved N-termini and C-termini of the protein that amplify most of the flagellin gene; this generates amplicons of 1·02 kb and 1·25 kb for type a and b flagellins, respectively. The central part of the flagellin gene shows considerable variability. Digestion of the amplicons separately with seven restriction endonucleases (*Cfo*I, *Hae*III, *Mbo*I, *Msp*I, *Rsa*I, *Sal*I, and *Taq*I) followed by agarose gel electrophoresis, staining with ethidium bromide, and ultraviolet transillumination permits detection of restriction fragment length polymorphisms in the flagellin gene.[19] 13 different *P aeruginosa* flagellin gene variants have been reported.[19]

Results

Review of the case-notes showed that, in March, 1995, 92 (76·7%) of the 120 children were colonised with *P aeruginosa*. Disk susceptibility tests had shown that 65 (70·6%) of these colonised patients harboured isolates that were resistant to ceftazidime and other β-lactam antibiotics but sensitive to tobramycin and ciprofloxacin. Retrospective analysis of the patients' case-notes indicated that a ceftazidime-resistant *P aeruginosa* had first been detected in 1987. Since then, there has been a cumulative increase in ceftazidime-resistant *P aeruginosa*, with four to 12 newly colonised patients each year (table). The median age at acquisition was 7·8 years (range 0·3–18·2).

The results of the two genomic fingerprinting methods were concordant and showed that 55 of the 65 children with β-lactam-resistant *P aeruginosa* harboured the same

Year	Number of cases	
	New cases	Cumulat
1987	1	1
1988	4	5
1989	4	9
1990	12	21
1991	6	27
1992	8	35
1993	10	45
1994	10	55
1995	10	65

March, 1995, cohort.

Table: **Patients infected with ceftazidime-resistant and β-lactam-resistant *Pseudomonas aeruginosa*, 1987–95**

epidemic strain (figure). In addition, six of the 5 children harboured other *P aeruginosa* isolates, whic were unique for each patient. The remaining eigh children each harboured one or more distinct *P aeruginos* strains not found in other children. All but one of the 5 children had been colonised with a ceftazidime-sensitiv *P aeruginosa* before culture of the epidemic, resistan strain. A ceftazidime-resistant *P aeruginosa* that had bee isolated from one of them in 1988 had the same genomi

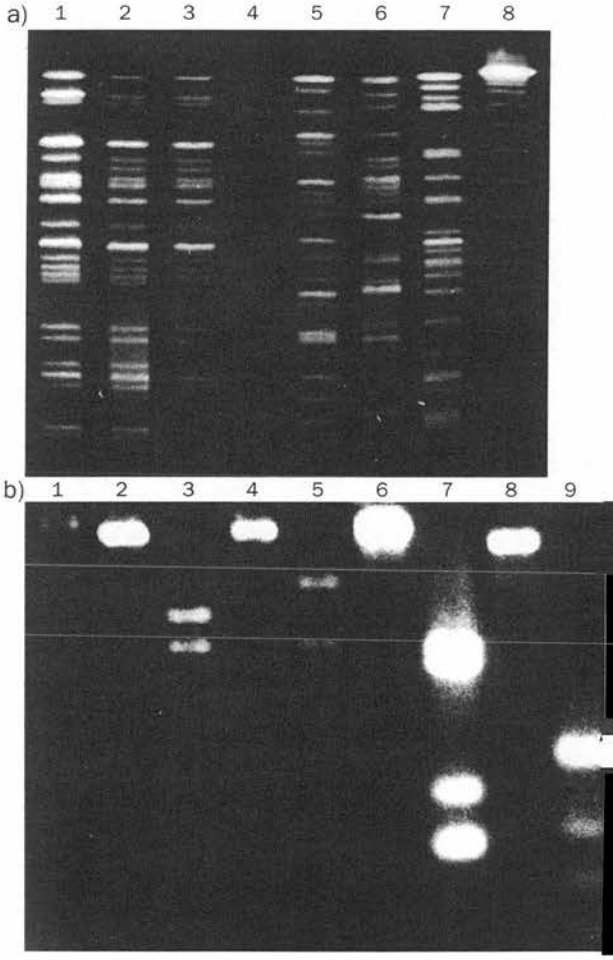

Figure: **Genotyping of β-lactam-resistant *P aeruginosa***

(a) chromosomal DNA digested with *Xba*I and separated by pulsed-field g electrophoresis. Lanes 1, 2, and 3 show epidemic strain; lanes 5, 6, an 7 show patterns produced by non-epidemic *P aeruginosa*; lane 8 is a DN size marker.

(b) DNA size marker (50–2000 bp) in lane 1; flagellin gene amplification product from epidemic strain (type b flagellin) in lane 2 and digested wit *Mbo*I in lane 3; flagellin gene amplified from a non-epidemic strain (type flagellin) in lane 4 and digested by *Mbo*I in lane 5; a third type b flagellin gene in lane 6 and digested with *Mbo*I in lane 7; a type a flagellin gene i lane 8 and digested by *Mbo*I in lane 9.

fingerprint as the epidemic strain. Review of the notes showed that only three of the 92 children colonised with *P aeruginosa* had never received any intravenous ceftazidime; two of these three children carried the epidemic strain. The epidemic strain was resistant to ceftazidime (MIC_{90} >32 mg/L), azlocillin (>64 mg/L), and imipenem (>32 mg/L); it was susceptible to tobramycin (0·5 mg/L) and ciprofloxacin (0·05 mg/L). (MIC_{90} is the lowest antibiotic concentration that inhibits the growth of 90% of isolates.)

Discussion

The proportion (76·7%) of our clinic population colonised with *P aeruginosa* is typical of many other CF centres. By contrast, the observation that 70·6% of these patients harboured ceftazidime-resistant and β-lactam-resistant *P aeruginosa* is higher than the figure of 14% expected from previous surveillance studies in our centre[20] and of 18·6% from national studies.[21] A major objective of our study was to see whether acquisition of β-lactam-resistant *P aeruginosa* reflected antibiotic-induced or selected resistance in individual patients or cross-infection of individual patients with a single resistant strain. The latter proved to be the case for 55 (85%) of the patients harbouring β-lactam-resistant *P aeruginosa*. Our results also raise concern about antipseudomonal therapy—in particular, therapy with a single antipseudomonal agent. We speculate that the introduction of ceftazidime monotherapy in our centre in 1986 as standard antipseudomonal treatment for respiratory exacerbations might have been a contributory factor to the development of the high prevalence of β-lactam resistance.

The importance of our findings is two fold. First, concordant data based on two highly discriminatory genomic fingerprinting techniques showed the presence of identical strains of *P aeruginosa* from 55 out of a cohort of 65 patients. This observation provides the first unequivocal evidence of extensive spread of *P aeruginosa* in a CF centre. Second, that most isolates of the epidemic strain were resistant to ceftazidime and to other β-lactam antibiotics is worrying. Retrospective analysis of case-notes showed that ceftazidime-resistant and β-lactam-resistant *P aeruginosa* had first been reported in our centre in 1987. Unfortunately, isolates from that year were not available to see whether they were clonally related to the epidemic strain, although typing of one stored isolate indicated that the epidemic strain was present in 1988. Again, because there were no stored isolates, we could not directly ascertain whether colonisation with a ceftazidime-resistant *P aeruginosa* between 1988 and 1995 involved the same epidemic strain.

Previous studies have shown that combination therapy with ceftazidime and an aminoglycoside has no clinical advantage over ceftazidime monotherapy[22] and that the prevalence of ceftazidime-resistant *P aeruginosa* in CF patients is unrelated to age but associated with prolonged *P aeruginosa* colonisation, a low Schwachman score, and increased use of ceftazidime.[20]

Why was there such a high prevalence of β-lactam-resistant *P aeruginosa* in our centre? The use of ceftazidime monotherapy might have caused induction and selection of β-lactamase-producing *P aeruginosa*. This possibility is supported by the observation that most patients initially harboured ceftazidime-sensitive *P aeruginosa*. However, the precise mechanism of β-lactam resistance remains to be determined and, if the hypothesis were true, each patient would have been colonised by a distinct strain of *P aeruginosa*. An alternative hypothesis is that each patient might have acquired a sensitive form of the epidemic strain from a common source, and that resistant forms were subsequently induced or selected as a result of antimicrobial therapy. The high number of colonised patients makes this hypothesis unlikely, though we do not reject it entirely. Extensive microbiological surveillance of the ward and clinic environment, including respiratory function equipment, did not detect the epidemic strain. Moreover, retrospective identification of the epidemic strain in the hospital environment could have resulted from contamination by colonised patients. The most likely explanation is a combination of induction and selection of ceftazidime resistance and person-to-person spread of a highly transmissible strain of *P aeruginosa*.

Since identifying the epidemic strain, we have instituted measures to diminish its spread. Intravenous antibiotic therapy is given at home to most patients supported by a visiting nurse. Patients harbouring the β-lactam-resistant *P aeruginosa* are isolated while in hospital, and we discourage social mixing of patients. Our antibiotic policy now is to treat patients who have ceftazidime-sensitive *P aeruginosa* with ceftazidime and tobramycin and those who have the resistant strain with colistin and tobramycin.

In previous reports of the spread of multidrug-resistant *P aeruginosa* in Danish CF patients, Hoiby and colleagues[7,23] reported a substantial increase in resistance to ceftazidime and piperacillin and confirmed induction of β-lactamase as the major resistance mechanism. The mechanism of resistance in our epidemic strain is unclear.

We have confirmed the long-term spread of a β-lactam-resistant strain of *P aeruginosa* among CF patients attending the same clinic. It is also possible that the particular strain responsible represents an unusual highly transmissible form of *P aeruginosa* resembling highly transmissible lineages of *B cepacia*.[4] On the basis of previous studies suggesting that spread of *P aeruginosa* is uncommon and usually limited to CF siblings, we do not recommend that all patients colonised with *P aeruginosa* should be segregated. Rather, our results indicate that careful surveillance of the prevalence of antibiotic resistance and cross-infection in CF centres should have an important role in infection control and that antipseudomonal monotherapy should be considered with caution.

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